UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

GUSTAVO HAUBER GAMEIRO

A INFLUÊNCIA DO ESTRESSE SOBRE A NOCICEPÇÃO INDUZIDA NA ARTICULAÇÃO TEMPOROMANDIBULAR

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do título de Doutor em Odontologia, Área de Concentração em Fisiologia Oral.

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A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, em sessão pública realizada em 16 de Fevereiro de 2006, considerou o candidato GUSTAVO HAUBER GAMEIRO aprovado.

PROFa. DRa. MARIA CECILIA FERRAZ ARRUDA VEIGA

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PROF. DR. PAULO HENRIQUE FERREIRA CARIA

PROF. DR. EDUARDO DIAS DE ANDRADE

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SUMÁRIO

RESUMO

Estudos recentes têm investigado o papel dos fatores psicológicos nas desordens temporomandibulares (DTM). Entretanto, os mecanismos responsáveis pelas alterações nociceptivas induzidas pelo estresse não estão bem estabelecidos. Desta maneira, o objetivo deste estudo foi avaliar os efeitos do estresse agudo, sub-crônico e crônico sobre a nocicepção induzida pela injeção de formalina na articulação temporomandibular (ATM) de ratos. Foi avaliada a relação entre os níveis sangüíneos de adrenocorticotropina (ACTH), corticosterona, os níveis de ansiedade e as respostas nociceptivas registradas após os diversos protocolos de estresse. Os animais foram inicialmente submetidos a uma sessão de estresse agudo por contenção (15 min; 30min e 1h), ou expostos a um estresse sub-crônico (3 dias–1h/dia) ou crônico (40 dias–1h/dia). Logo depois, os animais foram **(1)** mortos imediatamente para coleta de sangue e mensuração hormonal por radioimunoensaio; ou **(2)** submetidos ao teste do labirinto em cruz elevado para avaliação da ansiedade; ou **(3)** submetidos ao teste da formalina na ATM para avaliação da nocicepção. Finalmente, foi avaliado o papel do sistema serotoninérgico e opióide nas alterações nociceptivas induzidas pelo estresse. Para isso, um inibidor seletivo da recaptação de serotonina (fluoxetina 10 mg/Kg) e um agonista opióide (morfina 1-5 mg/Kg) foram administrados antes da realização dos ensaios de nocicepção. Os resultados mostraram que todos protocolos de estresse aumentaram significativamente os níveis de ACTH ou corticosterona, bem como o comportamento de ansiedade. Em relação à nocicepção, os animais cronicamente estressados apresentaram aumento nas respostas nociceptivas (hiperalgesia). Nesse grupo ocorreu redução do efeito analgésico da morfina, indicando disfunção do sistema opióide endógeno. A fluoxetina teve efeito analgésico tanto no grupo estressado (hiperalgésico) quanto no grupo controle (não-estressado), porém o efeito foi maior no grupo estressado. Concluiu-se que a hiperalgesia induzida pelo estresse resultou das alterações nos sistemas serotoninérgicos e opióides, as quais podem explicar, pelo menos em parte, a importante ligação entre estresse e dor orofacial.

Palavras-chave: Estresse, Articulação temporomandibular, Dor facial

ABSTRACT

Recent studies have investigated he role of psychological factor in temporomandibular disorders (TMD). However, the mechanisms responsible for nociceptive changes induced by stress are not established. Thus, the aim of this study was to evaluate the effect of acute, sub-chronic and chronic stress on nociception induced by formalin injection in rats' temporomandibular joint (TMJ). The relation beetwen blood levels of adrenocorticotropin (ACTH), corticosterone, the levels of anxiety and nociceptive responses recorded after the various stress protocols was evaluated. Animals were initially submitted to one session of acute restraint stress (15 min; 30 min and 1 h), or exposed to sub-chronic stress (3 days-1h/day) or chronic stress (40 days-1h/day). After, animals were (1) killed immediately to collect blood for hormonal determinations by radioimmunoassay; or (2) submitted to the elevated plus-maze to evaluate anxiety; or (3) submitted to the TMJ formalin test to evaluate nociception. Finally, the role of serotoninergic and opioid systems in nociceptive changes induced by stress was evaluated. For this, the serotonin-selective reuptake inhibitor (fluoxetine 10 mg/Kg) and the opioid agonist (morphine 1-5 mg/Kg) were administered before the nociception tests. The results showed that all stress protocols increased significantly the levels of ACTH or corticosterone, as well as the anxiety behavior. In relation to nociception, the chronic stressed animals showed an increase in nociceptive responses (hyperalgesia). In this group, there was a reduction in the morphine analgesic effects, suggesting dysfunction in the endogenous opioid system. Fluoxetine had an analgesic effect in both stressed (hyperalgesic) and control groups (non-stressed), although the effect was more significant in the stressed-group. It was concluded that stress-induced hyperalgesia may result from changes in the serotoninergic and opioid systems, which can explain, at least in part, the important link between stress and orofacial pain.

Keywords: Stress, Temporomandibular joint, Facial pain

I. INTRODUÇÃO

Inúmeras investigações têm examinado a relação entre estresse psicológico e desordens temporomandibulares (DTM) (Grzesiak, 1991; Vanderas, 1994; Wexler & Steed, 1998). Foi observado que pessoas expostas a situações estressantes estão sob maior risco de ocorrência e progressão de DTM (Speculand *et al*., 1984), e pacientes com disfunção relatam que seus sintomas aumentam durante eventos estressantes (Suvinen *et al*., 1997). O efeito do estresse nas funções do sistema estomatognático ocorre por meio de complexas inter-relações no sistema nervoso central. Interação entre o sistema límbico e o centro de atividade motora permite a transformação de um processo emotivo e cognitivo em resposta motora (Bullock & Rosedahl, 1992), que na área do sistema estomatognático manifesta-se como aumento do tônus muscular. A tensão muscular que acompanha condições emocionais estressantes é um importante fator etiológico para muitos problemas disfuncionais e dolorosos (Parker, 1990). Além disso, a disfunção muscular induzida por estresse pode secundariamente produzir alterações na articulação temporomandibular (ATM), resultando em mudanças na biomecânica articular, microtraumas às cápsulas articulares e meniscos e alterações na percepção de dor (Uhac *et al*., 2003).

A analgesia induzida por estresse tem sido demonstrada tanto em humanos (Bandura *et al*., 1988; Droste *et al*., 1991) como em animais (Mogil *et al*., 1996; Wiedenmayer & Barr, 2000; Lapo *et al*., 2002). Em 1977, Chesher e Chan demonstraram que o choque nas patas (*footshock*) de camundongos produzia um efeito analgésico, o qual era antagonizado pela naloxona, um antagonista de receptor opióide. O *footshock* mostrou ser capaz de aumentar os níveis de peptídeos opióides endógenos (Akil *et al*., 1976). Subseqüentemente, diversos estressores incluindo o footshock, natação, imobilização, isolamento e restrição têm sido utilizados para o estudo da analgesia induzida por estresse. Os efeitos analgésicos induzidos por estes estressores são comparados àqueles causados pela morfina em doses de 5-10 mg/Kg, porém a duração desses efeitos é relativamente menor, desaparecendo aproximadamente dentro de 30 minutos (Snow e Dewey, 1983; Giradot & Holloway, 1984).

Embora os estudos anteriores tenham demonstrado os clássicos efeitos analgésicos do estresse, muitas pesquisas relatam que determinadas condições experimentais (estresse agudo e crônico) podem provocar hiperalgesia ao invés de analgesia (Vidal & Jacob, 1982; Satoh *et al.*, 1992; Quintero *et al*., 2000; Imbe *et al.*, 2004). Por exemplo, uma breve exposição a um estresse emocional, como a exposição a novos ambientes, produz uma hiperalgesia imediata e transitória (Vidal & Jacob, 1982), enquanto o estresse prolongado por contenção (40 dias) induz hiperalgesia que persiste por até 28 dias após a suspensão do estresse crônico (Torres *et al*., 2003). Os mecanismos relacionados à hiperalgesia de longa duração ainda não estão esclarecidos. É possível que esse aumento de percepção aos estímulos dolorosos estejam relacionados a alterações no eixo hipotálamo-hipófise-adrenal, nos receptores opióides ou em qualquer outro sistema responsável pela resposta de estresse. A deficiência na transmissão serotoninérgica central pode produzir sensibilização das vias de transmissão da dor, por isso o estresse crônico pode estar associado a aumentos na sensibilidade dolorosa (Quintero *et al*., 2000).

As divergências em relação aos efeitos do estresse sobre a nocicepção ocorrem, pelo menos em parte, devido ao fato de que a resposta de estresse depende de fatores como a natureza, a intensidade e a duração do estímulo estressor (Terman *et al*., 1986). Além disso, o estresse geralmente é acompanhado por estados emocionais, como a ansiedade e o medo (Mechiel Korte & DeBoer, 2003). Muitos trabalhos têm demonstrado que as alterações nos estados emocionais tanto de humanos (Barlow *et al*., 1996), como de animais (King *et al*., 1996) podem alterar fortemente a reatividade à sensação dolorosa. Por isso, um modelo experimental destinado ao estudo da relação entre dor e estresse precisa considerar as diversas variáveis, fisiológicas, psicológicas e comportamentais envolvidas em uma situação de estresse.

Nos estudos citados anteriormente, os testes utilizados para medir a nocicepção consistiam na aplicação de estímulos nocivos fásicos a tecidos superficiais, como por exemplo o *tail-flick*, no qual é determinado o tempo de latência para mover a cauda após a aplicação do estímulo. Não existem modelos experimentais em animais sobre o efeito do estresse em condições dolorosas profundas, as quais possuem características diferentes em relação às dores provenientes de tecidos cutâneos (Sessle & Hu, 1990).

Considerando a relação existente entre estresse e crises de dor facial (Suvinen *et al*., 1997) e também a capacidade do estresse em alterar a percepção e resposta à dor, estudos sobre os mecanismos das alterações nociceptivas induzidas pelo estresse nas dores profundas são relevantes para a pesquisa sobre a etiologia das desordens temporomandibulares.

II-PROPOSIÇÃO

Os objetivos do presente trabalho foram:

- Verificar o efeito do estresse agudo, sub-crônico e crônico sobre as respostas comportamentais nociceptivas induzidas pelo teste da formalina na ATM de ratos.
- Avaliar a relação entre os diversos protocolos de estresse, os níveis de ansiedade, os níveis sangüíneos de ACTH e corticosterona e as respostas comportamentais nociceptivas induzidas pelo teste da formalina na ATM.
- Avaliar a participação do sistema opióide e serotoninérgico nas alterações nociceptivas induzidas por situações estressantes.

O presente estudo foi realizado em formato alternativo, conforme deliberação da Comissão Central de Pós-graduação (CCPG) da Universidade Estadual de Campinas (UNICAMP) nº 001/98.

III- CAPÍTULOS

Artigo 1

"How do stressful experiences contribute to the development of orofacial pain?". Este artigo foi submetido à publicação no periódico Clinical Oral Investigations.

Artigo 2

"The effects of acute and chronic restraint stress on nociceptive responses induced by formalin injected in rat's TMJ". Este artigo foi publicado no periódico Pharmacology Biochemistry and Behavior *(Pharmacol Biochem Behav. 2005 Oct;82(2):338-44).*

Artigo 3

"Nociception- and anxiety-like behavior in rats submitted to different periods of restraint stress". Este artigo foi aceito para publicação no periódico Physiology & Behavior.

Review article

How do stressful experiences contribute to the development of orofacial pain?

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Abstract: Temporomandibular disorders (TMD) comprise the most common cause of chronic facial pain conditions, and they are often associated with somatic and psychological complaints including fatigue, sleep disturbances, anxiety and depression. For many health professionals, the subjectivity of pain experience is frequently neglected, even when the clinic does not find any plausible biologic explanation for the pain. This strictly biomedical vision of pain cannot be justified scientifically. The purpose of this study is to demonstrate, by original articles from the literature and recent studies conducted in our own laboratory, the biological processes by which psychological stress can be translated into the sensation of pain and contribute to the development of TMD. The role of the hypothalamic-pituitary-adrenal axis, the serotoninergic and opioid systems in the pathogenesis of facial pain is exposed, including possible future therapeutic approaches. It is hoped that knowledge from apparently disparate fields of dentistry, integrated into a multidisciplinary clinical approach to TMD will improve diagnosis and treatment for this condition, through a clinical practice supported by scientific knowledge.

Descriptors: stress, temporomandibular disorders, facial pain.

Running head: Oral Physiology- Orofacial Pain

Introduction

Temporomandibular disorders are musculoskeletal pain conditions characterized by pain in the temporomandibular joint and/or the masticatory muscles [1]. The clinical condition of TMD can also involve sounds during mandibular movement and limited mandibular movement [2]. TMD pain is the commonest symptom that compels patients to seek therapy. In the USA and Europe, chronic facial pain accounts for 40% of all chronic pain problems [3, 4]. In Brazil, the prevalence of TMD symptoms is between 40 -60% [5, 6]. Although the underlying cause of TMD remains poorly understood, it is widely recognized to be multifactorial, involving physiological, behavioural, and environmental factors. In dental research, dental occlusion and Para functional activities were the two etiologic factors that have received the most attention in epidemiological studies [7, 8]. The etiologic role of malocclusion, jaw position and biomechanical factors has been questioned. For example, various studies did not find association between occlusion and TMD (for review, see [9, 10, 11]). When such association was present, some studies revealed that occlusal factors were only weakly associated with TMD signs and symptoms [12, 13]. A prospective investigation over two decades into signs and symptoms of temporomandibular disorders indicates that a lateral forced bite between the retruded contact position and the intercuspal contact position and a unilateral crossbite deserve further consideration as possible local risk factors for development of TMD [13]. In relation to oral parafunctions, some experimentally induced habits can cause pain, similar to that related by patients with TMD [14, 15]. Although parafunctional clenching involves increased masticatory muscle activation [16], which can sometimes evoke pain [17], bruxism activity was not always

correlated with TMD pain [18]. Moreover, there are people classified as bruxers, who did not present history of pain in masticatory muscles [19, 20]. Therefore, it is difficult to establish any direct relation to prove that parafunctional activities can really cause TMD.

On the other hand, Laskin was the first to suggest that the main factors responsible for TMD are emotional instead of physical [21]. During the last decade, numerous investigations have been devoted to understanding the relationship between psychological stress and TMD [22, 23, 24]. Patients suffering from this condition report that their symptoms increase during stressful situations [25]. De Leeuw et al. (1994) consider that muscle dysfunction and accompanying pain are very often the result of stress induced muscular hyperactivity [26]. Stress induced muscular dysfunction may induce secondary changes in the temporomandibular joint (TMJ). Raised elevator tonus leads to increased intra-articular pressure in TMJ and alteration in the normal biomechanics, resulting in microtraumatic damage to the joint capsules and disk attachment. However, the studies that investigate psychological factors present mixed results. Some investigators related electromyographic changes in masticatory muscle baseline values between patients with TMD and control individuals [27, 28, 29] while others did not find significant differences in electromyographic activity baseline values between patients and controls [30, 31]. These inconsistencies may be probably due to different methodologies used.

The authors believe that both physical and psychological factors contribute to the onset and maintenance of TMD. The balance of these factors produces many individual differences in the perception of pain. More important than to argue in support of the supremacy of some etiologic factor (physical or psychological), is to understand to what

extent some factor is responsible, how it is involved and what can be done to alleviate the suffering of TMD patients.

The purpose of this article is to demonstrate the biologic process by which stressful experiences can influence pain perception, and thus, the development of TMD. The notion of the physiologic and pathophysiologic manifestations of stress system is described, including possible future therapeutic approaches.

Stress System - Physiology

Life, as a high-order dynamic equilibrium, is constantly in a state of threatened homeostasis, or stress. Thus, the forces that disturb homeostasis, the stressors, are counterbalanced by adaptive forces generated by the organism [32]. Both physical and emotional stressors set into motion central and peripheral responses, designed to preserve homeostasis [33]. Centrally, neural pathways are facilitated, which among other functions, mediate arousal, vigilance, cognition, as well as appropriate aggression, with concurrent inhibition of pathways that subserve vegetative functions, such as feeding and reproduction. Peripheral changes occur principally to promote an adaptive redirection of energy. Thus, oxygen and nutrients are directed to the central nervous system and the stressed body site [34].

It has to be borne in mind that not all states of stress are noxious. Selye made it clear when he coined the terms "eustress" and "distress". Hence, he believed that mild, brief, and controllable states of challenged homeostasis could actually be perceived as pleasant or exciting and could be positive stimuli to emotional and intellectual growth and development - it is notable that stress system activation occurs during both feeding and

sexual activity, for example. Selye believed that it was the more severe and uncontrollable situations of psychological and physical distress that led to frank disease states [35].

The central components of the stress system are located in the hypothalamus and the brainstem and include the corticotropin-releasing hormone (CRH) and the locus ceruleus-norepinephrine/autonomic sympathetic nervous systems [36]. The peripheral limbs of the stress system are the hypothalamic-pituitary-adrenal (HPA) axis, together with the efferent sympathetic/adrenomedullary system, and components of the parasympathetic system [32]. Central CRH and norepinephrine systems, together with peripheral secretion of large amounts of glucocorticoids and catecholamines, affect virtually every cell in the body [35]. Moreover, the stress system also interacts with other major central nervous system (CNS) elements, including the mesocorticolimbic dopaminergic system, the amygdala, the hippocampus, and the arcuate nucleus proopiomelanocortin (POMC) neuronal system [35]. The orchestrated interplay of several neurotransmitter systems in the brain underlies the characteristic phenomenology of behavioural, endocrine, visceral, autonomic, and immune responses to stress. These neurotransmitters include CRH, arginine vasopressine (AVP), opioid peptides, substance P, dopamine, serotonin, and norepinephrine. Therefore, an explanation about the functions of the neurotransmitters and hormones involved in the stress response is outside the scope of this article (for review, see Herman and Cullinan (1997) [37]). It is important to emphasize that most of the molecules mediating stress effects are the same as those associated with pain modulation (for review see Millan (2002) [38]), so the ability of stressful experiences to alter pain transmission and perception is obvious. Melzack postulated the existence of a pain neuromatrix [39] in which the experience of pain is produced by multiple influences and comprises a widely distributed

neural network with input from the body's stress regulation systems, including the hypothalamic-pituitary-adrenal (HPA) axis.

HPA axis - pathology

Dysregulation of the HPA has been demonstrated in several psychiatric stress-related disorders, such as depression [40] and post-traumatic stress disorder [41], which have a significantly higher prevalence among patients with TMD [42]. Stress system dysregulation can be expressed either as hyperfunction or as hypofunction. HPA axis hyperactivity occurs, for example, in melancholic depression [43], anorexia nervosa [44], obsessive-compulsive disorder [45], panic anxiety [33], and chronic active alcoholism [46]. On the other hand, stress system hypoactivation, rather than sustained activation, in which chronically reduced CRH secretion may result in pathologic hypoarousal, characterizes conditions such as fibromyalgia [47], seasonal depression [48], atypical depression [49], some forms of obesity [43] and the chronic fatigue syndrome [50]. In relation to TMD, it would appear that most TMD patients show HPA axis hyperactivity. Geissler [51] used biochemical evidence (urinary cortisol: creatinine ratios) to show that patients with TMD have higher urinary cortisol than normal individuals and therefore are under greater emotional stress. This study was carried out in patients who had been rendered free of pain or had only residual discomfort, so the stress factor would thus be emotional rather than pain-induced. Another recent study [52] indicated very high daytime cortisol levels in patients with facial pain, surprisingly much higher than those seen in depression or in fybromyalgia patients with generalized muscle pain [53]. It remains possible that facial

region pain represents a greater stimulus to HPA axis activation than pain elsewhere in the body.

Considering that pain itself acts as a strong activation of the HPA axis [54], it is possible that high levels of cortisol in TMD patients represent a physiologic response to chronic stress, with pain as a potential stressor, associated with chronically increased CRH or other HPA axis central mediators. Increased activation of the stress axis central components may result in hyperalgesia [55].

The study of the mechanisms involved in the relationship between stress and pain modulation in humans becomes more difficult, because of methodological, psychological, and ethical problems. On the other hand, animal models of nociception are very useful to understand the neural basis of the mechanisms involved in pain perception. The authors' laboratory is using an animal model of nociception, the TMJ formalin test [56], to evaluate the influence of stress on nociception induced by TMJ injury. The authors observed that rats submitted to chronic restraint stress (2 months) showed an increase in nociceptive responses, indicating that chronic stress could induce hyperalgesia [57]. The mechanism by which chronic stress produces hyperalgesia is not clear. In fact, more than one mechanism could be involved. The HPA axis is just one of the stress system biologic mediators. Next, the role of the serotoninergic and opioid systems in stress-induced hyperalgesia will be emphasized.

The role of serotoninergic system

Neurons that contribute to ascending nociceptive pathways involved in pain sensation are inhibited by descending serotoninergic and noradrenergic fibres, respectively

[58, 59]. Changes in the central serotoninergic system activities might, at least partly, explain the bidirectional changes in nociception (analgesia and hyperalgesia) seen after different stress conditions. For example, after acute exposure to different types of adverse psychological or physical stimuli, there is an increase in the extracellular concentrations of serotonin in several brain regions, especially in the raphe magnus [60]. Conversely, prolonged stress diminishes the efflux of serotonin in some brain structures known to be activated by stress, such as the amygdala and the lateral septum [61]. The magnitude of tonic inhibition of pain transmission within the spinal cord horn appears to be dependent on the behavioural state of the organism (depressed mood, anxiety, fear) [62]. The authors suggested that anxiety and stress can cause a deficit in the central serotoninergic transmission, which produces a sensitization of central pain relay pathways. First, stress was induced in rats by immobilization for 1 h (acute stress) or 2 months (chronic stress). This method is efficient to increase hormonal levels, as was detected by plasma corticosterone and ACTH determination by radioimmunoassay [57]. Next, the authors' test to evaluate nociception in the TMJ was used, as previously described [63]. Briefly, the rats received a 50 μl injection of diluted formalin (1.5 %) into the left TMJ region. The injections were given via a 30-gauge needle introduced into the TMJ capsule. After the TMJ injection, the rat was placed in the test chamber and nociceptive behavioural responses, characterized by rubbing the orofacial region (seconds) and flinching the head (number of times), were quantified for 30 min. A selective reuptake inhibitor, fluoxetine, was used to block the stress-induced hyperalgesia. Actually, fluoxetine administered 30 min before formalin had an analgesic effect analogous to that of morphine, observed in one

of the authors' studies [64]. These results are also consistent with correlational studies indicating that anxiety is related to increased pain reports in clinical settings [65,66].

Schreiber [67] found that fluoxetine relieved low back pain with efficacy similar to that of amitriptyline, and they suggested that fluoxetine could be an alternative for patients unable to tolerate tricyclic antidepressant side effects. The authors question the possibility of generalizing experimental findings to clinical settings, that is to say, it is too early to affirm that fluoxetine could be effective for treating TMD patients, even though some studies related that 5-HT re-uptake inhibitors have been associated with tooth-clenching or tooth-grinding [68]. Future studies should evaluate the possibility of dentists using fluoxetine to treat TMD patients.

Opioid Modulation

A major advance in the conception of the neural pain processing has occurred in the past decade. It has become clear that pain is not passively received by the nervous system, but is filtered and controlled (modulated) even at the first sensory synapse, by complex modulatory systems [38]. The existence of multiple pain-modulatory systems is used to clarify the bewildering profile of clinical observations resulting from various pain treatments. A major component of these systems is the intrinsic opioid systems, which are activated in stress situations and can diminish pain sensation [69]. For example, Maixner *et al.* (1990) [70] have shown that ischemic pain induced in the left arm was able to reduce pain sensation in patients suffering from acute dental pain. One important question is whether these endogenous inhibitory systems are functional in patients suffering from chronic facial pain. It is possible that chronic orofacial pain associated with TMD results

from diminished inhibitory systems in the central nervous systems. There is also evidence to support this idea. For example, 70 to 80% of TMD patients suffer from psychosomatic diseases, such as ulcers, headache, low back pain, asthma and dermatitis [21, 71]. The biochemical' contents of psychological and physiological stress are elevated in TMD patients when compared with controls [51, 52], suggesting that individuals with TMD are really under greater emotional stress than control individuals.

The authors' data from an experimental TMJ pain model indicate that endogenous inhibitory systems may be less effective under chronic stress conditions. The authors results demonstrate that repeatedly stressed rats display decreased morphine effects on nociception compared with non-stressed controls in the TMJ formalin test [57]. The tolerance of response to morphine observed in the authors study agrees with the hypothesis suggested by previous studies that chronic stress could modify opioid system activities (for review, see Drolet *et al.* (2001) [72]).

Conclusions and Future Therapeutic directions

Many patients with chronic facial pain improve with antidepressants, whether or not they have a comorbid depressive disorder [73, 3]. Antidepressants have the ability to modulate HPA axis activity and increase glucocorticoid receptors, though the mechanism by which this occurs is still unknown [74]. In view of the involvement of the HPA axis in depression and the deleterious effects of prolonged high cortisol levels, research into potential treatments of mood and pain disorders has focused on modulating the effects of

hypercortisolemia. A promising approach is the use of corticotropin-releasing hormone antagonists and there are several trials under way to test these agents in a variety of psychiatric disorders including depression. Another possibility is the use of glucocorticoid receptor antagonists to block any detrimental effects of the raised levels of circulating cortisol and also cause a compensatory up-regulation of glucocorticoid receptor number [75].

The authors concluded that the influence of stress on TMD is not as simple as suggested according to Laskin's theory, in which the stress evokes chronic recurrent muscular hyperactivity that progressively damages the joint, which in time becomes symptomatic [21]. The authors propose that stress can profoundly affect the biological processes of pain transmission and perception. Thus, inappropriate adaptational responses could be maladaptive and act as stressors themselves (orofacial pain is a strong stressor), feeding into a sustained vicious cycle. (fig.1).

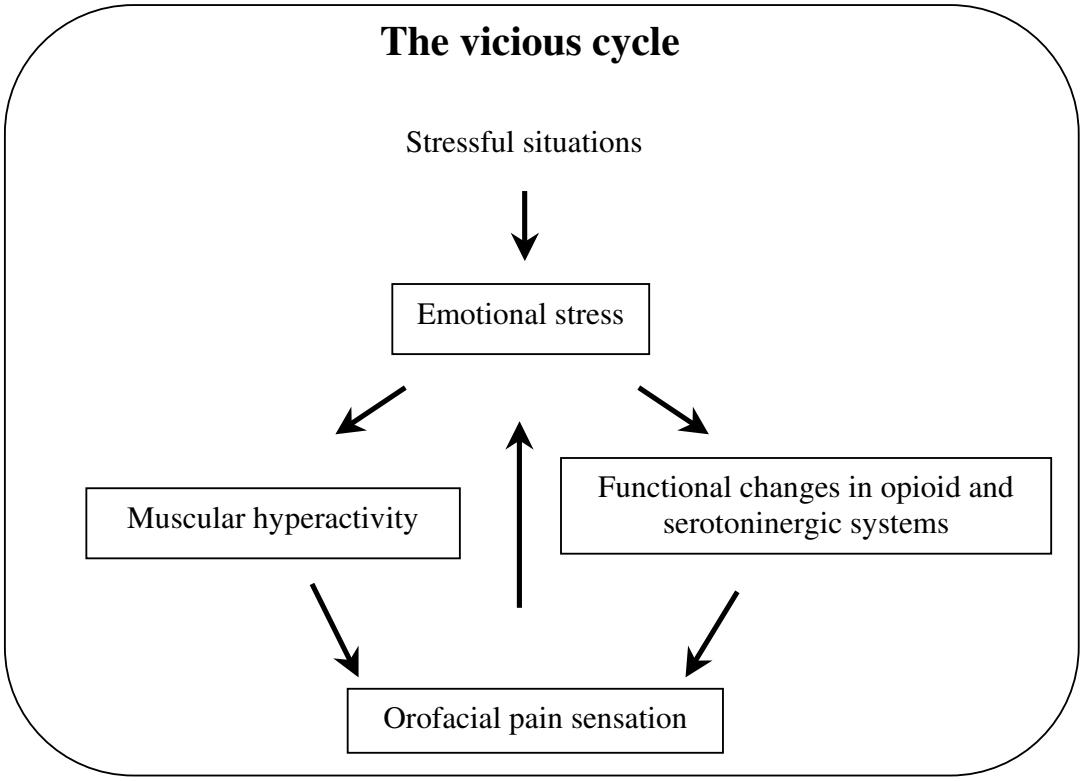

Fig 1- Diagram illustrating the cycle stress-pain-stress that can occur in TMD patients

In the authors' opinion, nociceptive controls exist not only for very stressful and/or nociceptive stimuli, but also for very mild stress that occur constantly i.e. situations occurring daily. This might explain why patients with TMD often have onset of their symptoms during periods of psychological stress (i.e. anxiety) and exacerbation of symptoms during periods of stressful situations [25].

Future research on stress-induced pain modulation should consider the multidimensionality of stress (physiologic and subjective experience) and its impact on the development of TMD. In addition to providing a more complete understanding of the

centrifugal control of pain, it is hoped that such information might suggest ways of relieving pain by less invasive means. The theoretical framework for testing the hypothesis that a dysregulation in the stress system can lead to TMD has been set in place, with the potential for improved understanding, diagnosis, and treatment of these disorders.

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Capítulo 2

The effects of restraint stress on nociceptive responses induced by formalin injected in rat's TMJ

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Abstract

It has been reported that stress can alter nociception from superficial tissues, such as skin and subcutaneous region. However, the influence of stress on an experimental deep nociception model is not understood. In this study, the temporomandibular joint (TMJ) formalin test was used to evaluate the effects of acute and chronic restraint stress on nociceptive responses in rats. Animals were initially submitted to one session of acute restraint stress (1 h) or exposed to chronic stress (40 days-1h/day). Then, animals were killed immediately to collect blood for hormonal determinations by radioimmunoassay, or submitted to the TMJ formalin test to evaluate nociception. Rats submitted to acute restraint presented a performance similar to unstressed controls in the TMJ formalin test, whereas chronically stressed rats showed an increase in nociceptive responses. After 40 days of restraint, morphine was injected i.p. (1, 5 mg/Kg or saline). The stressed rats displayed decreased morphine effects on nociception compared to unstressed controls. These findings suggest that repeated stress can produce hyperalgesia, which is, at least in part, due to alterations in the activity of opioid systems. This model may help elucidate the underlying neural mechanisms that mediate the effects of repeated stress on orofacial pain.

Keywords: **Stress; Hyperalgesia; Formalin test; Temporomandibular joint; nociception**

Introduction

Different effects upon the nociceptive response have been observed with exposure to acute and chronic stress in rats (Vidal and Jacob, 1982; Watkins et al., 1982; Bodnar, 1986; Kavaliers and Innes, 1992; Quintero et al., 2000). Acute exposure to a variety of stressors produces an immediate analgesia in several pain tests (Lewis et al., 1980; Urca et al., 1985; Terman et al., 1986; Vacarino and Kastin, 2001). Some studies, although, have reported that under some experimental conditions both acute and chronic stress can elicit hyperalgesia instead of analgesia (Satoh et al., 1992; Quintero et al., 2000, Quintero et al., 2003, Imbe et al., 2004). Repeated exposure to a cold environment (4^{o}C for 30 min every hour for 1 day) induces 3-day long mechanical hyperalgesia (Satoh et al., 1992). One hour restraint a day for 40 days produces thermal hyperalgesia, which persists for at least 28 days after suspension of the chronic treatment (Torres et al., 2003a). Finally, repeated non-noxious swim-stress (10-20 min a day for 3 days) elicits a delayed (after 24-48 h) and long-lasting (8-9 days) thermal and chemical cutaneous hyperalgesia (Quintero et al., 2000). Mechanisms regulating stress-induced changes in nociception include alterations in: endogenous opioid (Lewis et al., 1980; Przewlocki et al., 1987; Amit and Galina, 1988; Yamada and Nabeshima, 1995), serotoninergic (Quintero et al., 2000), adenosinergic (Torres et al., 2003b) and noradrenergic systems (Watkins and Mayer, 1982), as well as the hypothalamic-pituitary-adrenal (HPA) axis (Bodnar et al., 1979).

Although the precise mechanisms involved in the development of hyperalgesia observed after repeated stress are not well known, there are strong evidences that they could be related, at least in part, to alterations in the central or peripheral opioid activity (Gamaro et al., 1998; Torres et al., 2001a). The absence of novelty-induced antinociception, which has been attributed to opioid activation (Netto et al., 1987; Siegfried et al., 1987), in chronic stressed animals supports this theory. Therefore, one of the aims of the present work is to verify the effect of chronic restraint stress on morphine-induced antinociception, as measured by the TMJ formalin test.

The formalin test has been used to evaluate the effect of stressful stimuli in numerous experimental animal models, such as swim stress in mice (Carmody and Cooper,

1987; Vaccarino et al., 1992) and the exposure to a cat odour in rats (Lester and Fanselow, 1985). Our understandings of the influence of stress on nociception are largely based on experimental models of nociception in animals (Le Bars et al., 2001). Most of these models of nociception measure the output responses induced by superficial stimuli, for example tail-flick (Gamaro et al., 1998), hot-plate (King et al., 2003) and formalin injected in the paw (Aloisi et al., 1998). It is important to point out that deep pain conditions differ from the one evoked by superficial stimuli. There are different sensory disturbances in pain conditions involving deep tissues rather than cutaneous tissues (Sessle and Hu, 1990). Many deep craniofacial pain conditions, such as TMJ pain, are associated with manifestations of pain spread and referral (Sessle, 2002). Indeed, TMJ inflammation results in more robust changes in central nervous system when compared to perioral inflammation (Iwata et al., 1999). It is, nevertheless, poorly understood due in part to the limited options of experimental models available for the investigation of this condition.

Thus, considering that the nociceptive behavioral responses elicited by the injection of formalin into the TMJ represent a valid and reliable model of orofacial deep pain (Roveroni et al., 2001), the aim of this study was to evaluate the effects of acute and chronic restraint stress on the nociceptive responses induced by TMJ formalin test.

Methods

Animals:

Male Wistar rats (weighing 200-230 g at the beginning of experiment) obtained from Centro Multi-disciplinar de Bioterismo-Cemib, UNICAMP, Campinas, Brazil were used in this study. The rats were housed in groups of five and maintained in a temperature-controlled room (23 ± 1^{o}C) with a 12/12 light-dark cycle (lights on at 7:00 AM) and food and water were available *ad libitum*. Rats were adapted to the testing apparatus and handled prior to behavioral testing. Procedures were performed between 08:00 and 15:00 h. The study was conducted in accordance with the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983).

Stress exposure:

The animals were stressed by restraint 1 h daily, 5 days per week for 40 days in the chronic model (Ely et al., 1997). In the acute model, there was a single exposure (Gamaro et al., 1998). Restraint was carried out by placing the animal in a plastic restraint device (adjustable in size depending on the animal's weight) for 1 h. The area of the tube could be adjusted individually to each rat with a mobile inside wall and the tube was held firmly in place with Velcro straps. There was a 1 cm hole in the far end for breathing. The control group was not submitted to restraint. The immobilization procedure was carried out in a separate quiet room between 10:00 and 12:00 h.

Hormonal assays:

Plasma corticosterone and ACTH levels were determined by radioimmunoassay (RIA) after plasma extraction using ethanol or silic acid (Castro et al., 1995), respectively. The rats were decapitated immediately after the last stress session and the whole blood was collected. The time interval between the stress procedure and manipulations until sacrifice were strictly maintained similar (30 sec.) among the different groups (acute restraint group n=8; chronic restraint group n=8; acute control group n=8; chronic control group n=8).

Testing procedure for TMJ pain:

The design of this study follows that used by Roveroni et al. (2001). Testing sessions took place between 08:00 and 15:00 h in a quiet room maintained at 23 ± 1^{o}C. Immediately after the period of stress procedures, each animal was lightly anesthetized by inhalation of halothane to allow the TMJ injection.

Rats received a 50-μl injection of formalin diluted in saline (1.5 %) into the left TMJ region. The injections were performed via a 30-gauge needle introduced into the TMJ capsule. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50 μl) previously filled with formalin 1.5%.

Following the TMJ injection, the rat was placed in the test chamber (30 X 30 X 30 cm mirrored-wood chamber with glass at the front side) and nociceptive behavioral responses characterized by rubbing the orofacial region (amount of time-seconds) and

flinching the head (number of head flinches) were quantified for 30 min (10 blocks of 3 min). Considering that the flinching of the head behavior followed a uniform pattern of 1 s in duration, each flinching was expressed as 1 s. The combination (sum) of both behaviors provides a better measure of pain intensity than any single behavior (Roveroni et al., 2001; Gameiro et al., 2003). An investigator, who was blind to the rat's group assignment, made the analysis of the behaviors.

At the end of each experiment, Evans blue dye (0.1%, 5 mg/Kg) was injected systemically (via penile vein) in order to confirm the TMJ injection site at post-mortem, as previously described (Hass, 1992) by the visual examination of formalin-induced plasma extravasation of Evans blue dye bond to plasma protein.

Drug treatments:

In order to evaluate the role of endogenous opioids in nociceptive changes induced by stress, one opioid antagonist (naloxone) and one agonist (morphine) were used. In experiment 1, naloxone 10 mg/Kg (Vissers et al., 2004) was administered i.p. immediately after the acute restraint stress (1h) and before the TMJ formalin test. In experiment 2, the animals were submitted to chronic stress as described above. After 40 days of treatment (control group was left undisturbed in their home cage), the rats were injected i.p. with morphine 1.0 mg/Kg (Torres et al., 2003a), 5.0 mg/Kg (D'amato et al., 1999) or saline (n= 6/group) 30 min before the administration of formalin 1.5% into the TMJ. Morphine sulfate was dissolved in 0.9% saline and administered i.p. immediately after the last stress session in a volume of 1.0 ml/Kg.

Statistical analysis:

Statistical analysis of plasma corticosterone and ACTH data were performed using Student's t-test. Data were previously transformed to square-root or log, as indicated by the program SAS (version 8.2 for windows). The sum of rubbing and flinching responses exhibited by each animal was computed. The comparison between two groups was made by Student's t-test. The comparison of more than two groups (morphine effect analysis) was made by two-way analysis of variance (ANOVA). All values are given as mean +/-

standard error of the mean (SEM). A level of 5% was taken as evidence of statistical significance. Data were analyzed using SAS (version 8.2 for windows) by Institute Inc., Cary, NC, USA-licensed to Universidade Estadual de Campinas.

Results

Effects of stress procedures on plasma corticosterone and ACTH levels:

This experiment was carried out to define the efficacy of restraint in inducing stress-like hormonal modifications in the acute and chronic groups. There was a significant increase in plasma corticosterone (p<0.0001, t-test, Fig. 1A) and ACTH levels (p=0.0011, t-test, Fig. 1B) after a single restraint session for 1. The chronically stressed rats showed higher levels of corticosterone than control animals (p=0.0261, t-test, Fig. 2A). However, there was no difference in plasma ACTH levels between chronically stressed vs. control rats (p=0.4134, t-test, Fig.2B).

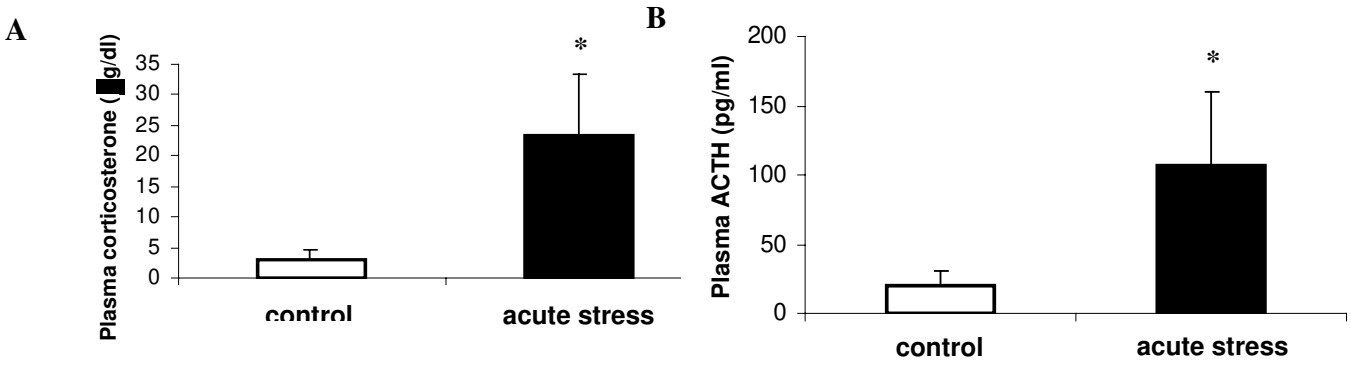

Fig 1A. Plasma corticosterone level after a single restraint session (1 h). Each data point represents mean ± SEM from 8 rats. The vertical bars indicate the standard error of the means. Data were analyzed using Student`s t-test. (*) Indicates significant difference compared with the control rats at p<0.0001. **Fig 1B.** Plasma ACTH level after a single restraint session (1 h). Each data point represents mean ± SEM from 8 rats. The vertical bars indicate the standard error of the means. Data were analyzed using Student`s t-test. (*) Indicates significant difference compared with the control rats at p=0.0011.

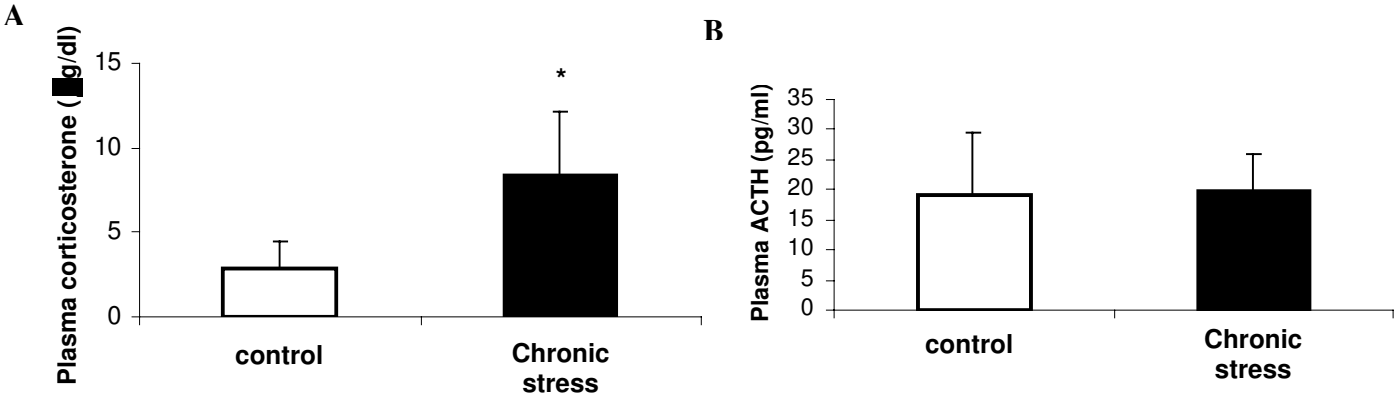

Fig 2A. Plasma corticosterone level after the last session of chronic stress (8-week). Each data point represents mean ± SEM from 8 rats. The vertical bars indicate the standard error of the means. Data were analyzed using Student`s t-test. (*) Indicates significant difference compared with the control rats at p=0.0261. **Fig 2B.** Plasma ACTH level after the last session of chronic stress (8-week). Each data point represents mean ± SEM from 8 rats. The vertical bars indicate the standard error of the means. Data were analyzed using Student`s t-test. There was no statistical difference between control and stressed groups (p=0.4134).

Effect of acute stress on nociceptive behavioral responses:

The exposure to a single restraint session for 1 h did not affect the nociceptive responses evoked by formalin 1.5% injected in TMJ of rats (Fig.3). There was no statistical difference (p=0.125) between the control group (non-stressed) and the stressed group.

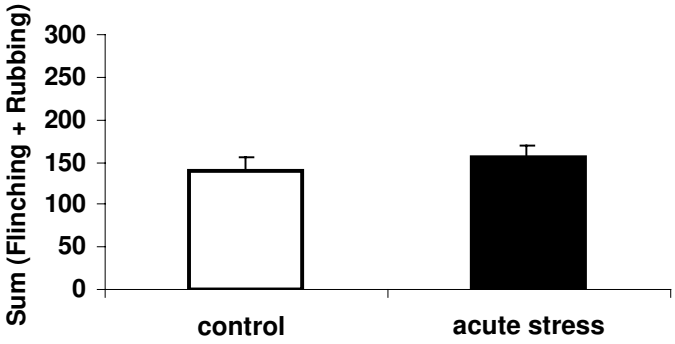

Fig 3. Sum of flinching and rubbing behaviors recorded in formalin-treated animals (50 μl, 1.5%) previously submitted to 1 h of restraint (n=6) or left undisturbed in their home cage (n=6). Each column represents the mean. Error bars indicate the SEM. No significant differences were found in nociceptive responses for control vs. stressed group (p=0.125, t-test).

Effect of chronic stress on nociceptive behavioral responses:

Results are shown in Fig. 4. Immediately after the last restraint session (1 h /40 days), the chronically-stressed animals were hyperalgesic. A statistically significant increase in the nociceptive behavioral responses was observed in the stressed group when compared with the control group ($p<0.05$, *t*-test).

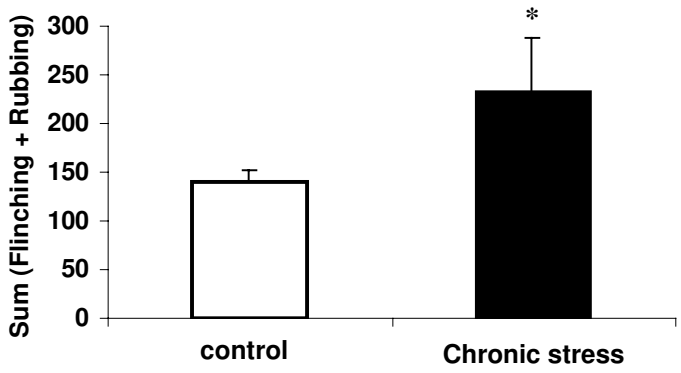

Fig 4. Sum of flinching and rubbing behaviors recorded in formalin-treated animals (50 μl, 1.5%) previously submitted to chronic stress ($n=6$) or left undisturbed in their home cage ($n=6$). Each column represents the mean. Error bars indicate the SEM. (*) Significant difference between the control and stressed group ($p<0.05$, *t*-test).

Effect of chronic restraint stress on rubbing spontaneous behaviors:

We also evaluated the spontaneous rubbing in order to exclude the possibility of an increased motor behavior induced by the chronic stress procedure. The chronic stressed rats exhibited a similar behavior than those of the control group (non-stressed) when saline was administered in the rat's TMJ ($p=0.7488$, Mann-Whitnet test, Fig.5).

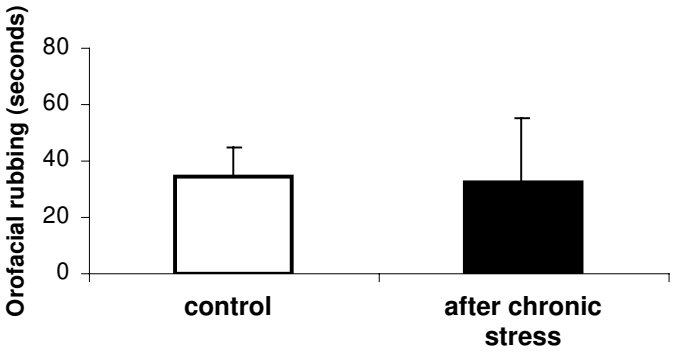

Fig 5. Duration of the orofacial rubbing behavior in rats previously submitted to chronic stress (*n*=6) or left undisturbed in their home cage (*n*=6). Each column represents the mean. Error bars indicate the SEM. There was no statistical difference between control and stressed groups (*p*=0.7488, Mann-Whitney test).

Effect of naloxone on nociception in rats submitted to acute restraint stress:

After one hour of immobilization, the injection of naloxone evoked an increase in nociceptive behaviors (180,69 ± 45,29), when compared with saline (123,14 ± 16,53). The increase in the sum of nociceptive behaviors (flinching + rubbing) was statistically significant (*p*=0.0489, t-test, Fig.6).

Fig 6. Effects of naloxone or saline on formalin-treated animals (50 µl, 1.5%) previously submitted to acute restraint stress (*n*=6/group). Each column represents the mean. Error bars indicate the SEM. (*) Indicates significant difference compared with the saline group (*p*=0.0011, t-test).

Effect of morphine on nociception in repeatedly-stressed and control rats:

Results referring to the analgesic effect of morphine are shown in Fig. 7. ANOVA revealed significant interaction between stress and morphine (p=0.003). Pos-hoc tests (Tukey) revealed that morphine administration produced a significant reduction of nociceptive behavioral responses in the control group (non-stressed). Morphine 1 mg/Kg reduced the nociceptive responses 30 min after the administration (p<0.05), and morphine 5 mg/Kg also had this effect (p<0.05). In the stressed group, morphine had an effect only at the dose of 5 mg/Kg (p<0.05) when compared to the saline group.

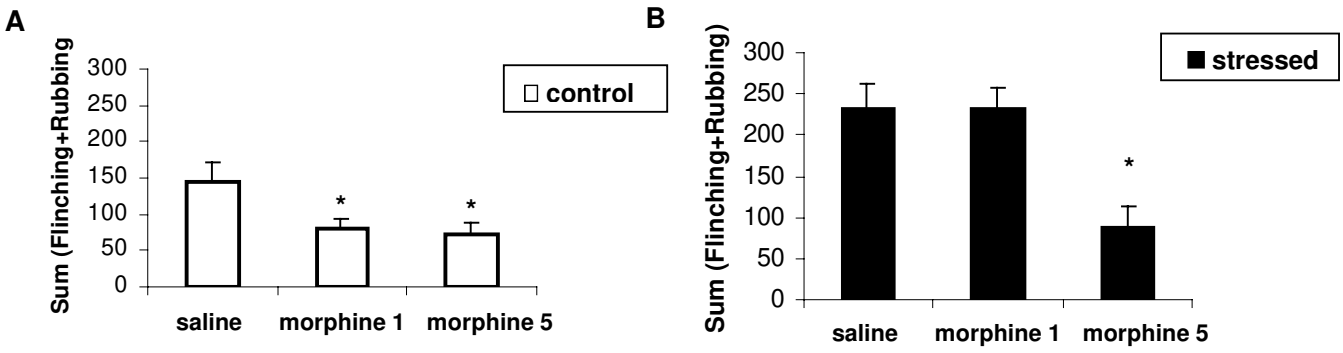

Fig 7. Sum of nociceptive responses to morphine (1 or 5 mg/Kg, i.p.) or saline after 40 days chronic restraint stress. *Panel A:* control groups (n=6/group); *Panel B:* stressed groups (n=6/group). Each column represents the mean. Error bars indicate the SEM. (*) Significant difference compared to saline group (p<0.05, ANOVA + Tukey).

Discussion

A variety of environmental and/or stressful stimuli have been shown to elicit analgesia, a phenomenon often referred to as stress-induced analgesia (SIA) (Amir and Amit, 1978; Watkins et al., 1982; Furuta et al., 2003). In the present study, a single exposure (1 h) to restraint stress did not reduce the nociceptive behavioral responses evoked by nociceptive chemical stimulation (formalin 1.5%) of the rat's TMJ. The ability

of the procedure to induce stress was confirmed by higher corticosterone and ACTH levels in restraint rats than those of control rats. One effect of acute stress exposure is a reduction of reflex responses that include tail or hinpaw withdrawal and licking in rats (Bodnar et al., 1980; Lewis et al., 1980; Gamaro et al., 1998). Although most of these responses involve a spinal-brain stem-spinal loop and appear to be purposeful, they do not depend upon cortical processing of nociceptive signals that result in pain perception (Mauderli et al., 2000; Vierck et al., 2002). King et al. 2003 showed that acute stress diminishes reflex responses to nociceptive input while enhancing operant responding to the same stimuli (nociceptive thermal stimuli), suggesting that stress induced hyporeflexia can coexist with stress induced hyperalgesia. According to these findings, we speculate that a single restraint session did not induce an analgesic effect on rats submitted to the TMJ formalin test, which evokes nociceptive responses that have an organization different from those related to innate reflexes, for example tail flick response that can be modulated directly at spinal levels (King et al., 2003). Moreover, the absence of stress-induced analgesia in our model may be related to the different site of formalin injection. As described in the introduction, nociceptive response evoked by cutaneous stimuli differs from the one evoked by deep stimuli. The discrepancy between nociception models in their susceptibility to modulation by stress is evident not only in the present results, but also in the partial and transient analgesic effects found in other studies employing the formalin test (Amir and Amit, 1979; Fuchs and Melzack, 1996; Aloisi et al., 1998).

The increase in nociceptive behavioral responses produced by chronic restraint stress has important implications in relation to other studies that have reported a hyperalgesic effect after exposure to a variety of stressors (Satoh et al., 1992; Quintero et al., 2000; Torres et al., 2003a,b). The present study confirmed the previously reported results for nociceptive responses, using an experimental model for the study of nociception from deep tissue injury: the TMJ pain. Although an extensive literature has reported the relationship between stress and chronic facial pain (Grzesiak, 1991; Vanderas, 1994; Korszun, 2002), little is known about the physiopathology of neural mechanisms that mediates the effects of repeated stress on pain sensitivity and affective states. The development of experimental models such as the present one may provide further

information about the mechanisms involved in these painful conditions and may be used to test the efficacy of drugs. In the current study, we were able to induce an increase in nociceptive behaviors following a repeated restraint stress procedure. In agreement with our results, previous studies have also found that chronic stress can elicit hyperalgesia rather than hypoalgesia (Lewis et al., 1980; Quintero et al., 2000; Torres et al., 2003a,b). Previous works have suggested that, when animals are repeatedly submitted to the same stressor, some behavioral and physiological consequences of stress exposure are reduced (habituation). For example, ACTH or corticosterone levels are reduced after repeated exposure to the same stressor (Marti and Amario, 1998; Torres et al., 2001b), although negative results have been reported (Dal-Zotto et al., 2000). In our model, corticosterone and ACTH levels were reduced after the end of stress session in 8-week restraint rats. However, the ability of the procedure to induce stress was confirmed by higher corticosterone levels in 8-week restraint rats than those of control rats. We also evaluated the spontaneous rubbing in order to exclude the possibility of an increased motor behavior induced by the chronic stress procedure. The chronic stressed rats exhibited a similar behavior than those of the control group (non-stressed) when saline was administered in the rat's TMJ. This result suggests the increase of flinching and rubbing behaviors is a hyperalgesic effect induced by chronic stress. The mechanism trough which repeated stress produces hyperalgesia is not clear; in fact, more than one mechanism could be involved. Satoh et al., 1992 suggested that mechanical hyperalgesia induced by prolonged cold stress involves peptide-containing primary afferents (substance-P and calcitonin-gene-related peptide). Quintero et al., 2000 showed that the increased thermal and chemical nociception observed after sub-chronic swimming stress might be mediated by changes in the activity of the central serotoninergic system. Torres et al. 2003b suggested that repeated restraint stress could induce an adaptative response in chronically stressed rats, which can lead to a desensitization of adenosine receptors. In other study, Torres et al. 2003a also showed that chronically stressed rats displayed decreased morphine effects on nociception.

In the last experiment, we tested control and repeatedly restrained rats injected with morphine (1 and 5 mg/Kg) in the TMJ formalin test. Our results demonstrate that repeatedly stressed rats display decreased morphine effects on nociception compared to

non-stressed controls. Although it has been described that morphine induces analgesia in a dose-related manner, in the present work it was not observed any difference between the two doses of morphine administrated in the control group (non-stressed). This discrepancy may be due to the different nociception assay used. We know that nociceptive transmission and modulation are different even when distinct superficial nociceptive essays are used (Fang and Proudfit, 1998). The stressed group needed an increased dose to show the classic analgesic effect of morphine. This change in sensitivity to morphine may be result of alterations in treatment-induced peptides release, i.e., persistent activation of opiate peptide receptors by endogenous opioids released during restraint stress could lead to receptor down-regulation, but it is possible that interactions with other released neurotransmitter could induce these effects, for example, serotonin, glutamate, adenosine and other opioid receptor systems have also been involved (Fitzgerald et al., 1996; Torres et al., 2003b). The tolerance of response to morphine observed in the present study agrees with the hypothesis suggested by previous studies that chronic restraint stress could modify the activity of opioid systems (for review, see Drolet et al., 2001). Changes in the analgesic effect of morphine observed in stressed rats might be due to alterations in central or peripheral opioid receptors, both in their affinity or number, or these changes might be due to alterations in other neuro-transmitter or hormonal systems able to interact with these receptors. Omiya et al., 2000 showed that hypofunction of the supraspinal mu-opioid receptor may explain the hyperalgesic effect of repeated cold stress loading in mice. Since morphine exerts its antinociceptive effects primarily through mu-opiate receptor subtype, the altered responses observed in animals submitted to TMJ formalin test after chronic stress might be due to changes at the level of these receptors. Future studies should evaluate the activity of the opioid receptors in this model. We suggest the influence of endogenous opioids released during chronic stress on the development of tolerance to morphine antinociceptive effects. This conclusion was based in the fact that restraint stress can release endogenous opioids, as was observed by the effect of naloxone on the augment of nociceptive responses in rats submitted to acute stress. In this case, it was expected that acute stress would reduce formalin-induced nociception, a finding not observed in our study. We believe that, in our model, the effects of endogenous opioids were

counterbalanced by the enhance in pain perception evoked by stress-induced-anxiety. Studies have shown that hyperalgesia is elicited by some experimental conditions (Cornwall and Donderi, 1988; Al Absi and Rokke, 1991; Meagher et al., 1998). In our laboratory, we have demonstrated that a single exposure to restraint stress (1 h) induced a high level of anxiety in the elevated-plus-maze (data not shown). This factor could also be determinant in the absence of stress-induced-analgesia. Continued research concerning the mechanisms of stress-induced hyperalgesia may be relevant to the study of the etiology of chronic pain disorders, like the temporomandibular disorder.

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Capítulo 3

Nociception- and anxiety-like behavior in rats submitted to different periods of restraint stress

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Abstract

The aim of this study was to evaluate the effect of acute, sub-chronic and chronic stress on nociception induced by formalin injection in rats' temporomandibular joint (TMJ). It was evaluated the relation between blood levels of adrenocorticotropin, corticosterone, the levels of anxiety and nociceptive responses recorded after different stress protocols. Animals were initially submitted to acute restraint stress (15; 30 min and 1 h), or exposed to sub-chronic (3 days-1h/day) or chronic stress (40 days-1h/day). Then, animals were (1) killed immediately to collect blood for hormonal determinations; or (2) submitted to the elevated plus-maze to evaluate anxiety; or (3) submitted to the TMJ formalin test to evaluate nociception. It was also evaluated the role of serotoninergic and opioid systems in nociceptive changes induced by stress. For this, the serotonin-selective reuptake inhibitor

(fluoxetine 10 mg/Kg) and the opioid agonist (morphine 1-5 mg/Kg) were administered before the nociception test. All stress protocols significantly raised the levels of ACTH or corticosterone, as well as the anxiety behavior. In relation to nociception, the chronic stressed animals showed an increase in nociceptive responses (hyperalgesia). In this group, there was a reduction in the morphine analgesic effects, suggesting dysfunction in the endogenous opioid system. Fluoxetine had an analgesic effect in both stressed and control groups, although this effect was more evident in the stressed group. It was concluded that stress-induced hyperalgesia may result from changes in the serotoninergic and opioid systems, which can explain, at least in part, the important link between stress and orofacial pain.

Keywords: Stress, Anxiety, Temporomandibular disorders, Facial pain

Introduction

An extensive literature has shown that acute exposure to a variety of stressors produces an immediate analgesia in several pain tests [1, 2, 3, 4]. Prolonged stress can also evoke analgesia [5]. However, some studies have reported that under some experimental conditions both acute and chronic stress can elicit hyperalgesia instead of analgesia. For example, rats exposed to acute and chronic restraint stress exhibit elevation and reduction of tail flick latencies, respectively [6]. Similarly, acute restraint stress reduced the duration of lick/guard responses to nociceptive input (analgesic effect), while the same acute stress for the same animals increased sensitivity to thermal stimulation, as assessed by learned escape responses (hyperalgesic effect) [7]. Taken together, these results reveal that the types of stressor, its intensity, duration, as well as the type of the nociceptive model used, affect not only the potency of analgesic or hyperalgesic effect but also the neuronal mechanisms responsible for them. The literature suggests that the stress-regulatory circuit activated by a particular stressor is crucially dependent on stimulus attributes [for review, see 8].

One factor that is particularly important is the emotional state induced by stress. For example, anxiety can produce hypervigilance which should increase attention to pain in human subjects, thereby amplifying its perceived intensity [9]. Recent work has shown that temporomandibular disorders (TMD) patients show increased stress, depression, anxiety and somatization compared with healthy controls [10, 11]. Many of the current treatments for these diseases utilize drugs that increase the levels or activity of the biogenic amine (e.g. serotonin, norepinephrine, dopamine) class of neurotransmitters. For example, fluoxetine, a specific serotonin-reuptake inhibitor (SSRI) that blocks the activity of serotonin transporter and increases the levels of 5-HT in the synaptic cleft, can be an effective treatment for depression and anxiety [12]. Although recent studies have investigated the role of psychological factors in TMD, the mechanisms responsible for nociceptive changes induced by stress are not established. The existence of multiple pain-modulatory systems is used to clarify the bewildering profile of clinical observation resulting from various pain treatments. A major component of these systems is the intrinsic opioid systems, which are activated in stress situations and can diminish pain sensation. For example, Maixner *et al.* [13] have showed that ischemic pain induced in the left arm was able to reduce pain sensation in patients suffering from acute dental pain. One important question is if these endogenous inhibitory systems are functional in patients suffering from chronic facial pain. It is possible that chronic orofacial pain associated with TMD result from inhibitory systems diminished in the central nervous systems. The absence of novelty-induced antinociception, which has been attributed to opioid activation [14, 15], in chronic stressed animals supports this theory. Thus, considering that the nociceptive behavioral responses elicited by the injection of formalin into the TMJ represent a valid and reliable model of orofacial deep pain [16], one of the aims of the present work was to evaluate the effects of different stress protocols on the nociceptive responses induced by TMJ formalin test. The role of serotoninergic and opioid systems in nociceptive changes induced by stress was also reported.

Methods

Animals:

Male Wistar rats (weighing 200-230 g at the beginning of experiment) obtained from Centro Multidisciplinar de Investigação Biológica -Cemib, Unicamp, Campinas, Brazil were used in this study. The rats were housed in groups of five and maintained in a temperature-controlled room (23 ± 1^{o}C) with a 12/12 light-dark cycle (lights on at 7:00 am) and food and water were available *ad libitum*. Rats were adapted to the testing apparatus and handled prior to behavioral testing. Procedures were performed between 08:00 am and 15:00 pm. The study was conducted in accordance with the ethical guidelines for investigations of experimental pain in conscious animals [17]. This research was approved by the institutional ethics committee in animal experimentation, according to the Brazilian College of Experimentation Guidelines.

Stress exposure:

The animals were stressed by restraint during 15 min, 30 min or 1 h in the acute model. In the sub-chronic model, animals were stressed by restraint 1 h daily, during 3 days. In the chronic model, animals were stressed by restraint 1 h daily, 5 days per week for 40 days [18]. The stress procedure in the chronic model consisted in 5 days of stress + 2 days of rest until 60 days. Thus, the protocol finished when 40 stress days were summed up. This protocol follows the design used by Gamaro et al., 1998 [19]. Restraint was carried out by placing the animal in a plastic restraint device (adjustable in size depending on the animal's weight) for 1 h. The area of the tube could be adjusted individually to each rat with a mobile inside wall and the tube was held firmly in place with Velcro straps. There was a 1 cm hole in the far end for breathing. The control groups were not submitted to restraint and were handled during the same time that their respective experimental groups. The control rats were handled in a quiet room once every day (handling comprised picking up each rat for a short period of time and then returning it to its home cage) during the days according to experimental stress procedures: in the acute model (15;30;60 min), control rats was handled just one time. In the sub-chronic model (3 days), control rats was handled (once a day) for 3 days. In chronic model, control rats were handled (once a day) until 40 stress

days were summed up. The restraint procedure was carried out in a separate quiet room between 10:00 and 12:00 am.

Hormonal assays:

Plasma corticosterone and ACTH levels were determined by radioimmunoassay (RIA) after plasma extraction using ethanol or silic acid [20], respectively. The rats were decapitated immediately after the last stress session and the whole blood was collected. The time interval between the stress procedure and manipulations until sacrifice was strictly maintained similar (30 sec.) among the different groups.

Evaluation of anxiety level:

The elevated plus-maze test was used to assess the anxiety level induced by different stress protocols. The elevated plus-maze was made of wood, according to specifications described in Morato and Brandão, 1997 [21]. The procedure was described elsewhere [22, 23]. Briefly, rats were placed in the central square facing a closed arm, and allowed to explore the elevated plus-maze for 5 min. Before the next rat was introduced, the maze was cleaned with a solution of 20% ethanol and dried. The conventional measures (percentage of open-arm entries, and the time spent on open arms) were recorded. The experimental sessions were recorded by a vertically mounted videocamera, linked to a monitor and VCR in an adjacent room. Videotapes were analyzed by highly trained observers who remained blind to treatment conditions. All tests were made immediately after the last stress session of the various protocols (15, 30, 60 min., 3 days and 40 days-n=10/group).

Testing procedure for TMJ pain:

The design of this study follows that used by Roveroni et al. 2001 [16]. After the last stress session, each animal was lightly anesthetized by inhalation of halothane to allow the TMJ injection. Rats received a 50-µl injection of formalin diluted in saline (1.5 %) into the left TMJ region. The injections were performed via a 30-gauge needle introduced into the TMJ capsule. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50 µl) previously filled with formalin 1.5%.

Following the TMJ injection, the rat was placed in the test chamber (30 X 30 X 30 cm mirrored-wood chamber with glass at the front side) and nociceptive behavioral responses characterized by rubbing the orofacial region (amount of time-seconds) and flinching the head (number of head flinches) were quantified for 30 min (10 blocks of 3 min). Considering that the flinching of the head behavior followed a uniform pattern of 1 s in duration, each flinching was expressed as 1 s in order to make simpler the quantification and representation of nociceptive behaviors, as previously described [16]. Moreover, the combination (sum) of both behaviors provides a better measure of pain intensity than any single behavior [16, 24]. An investigator, who was blind to the rat's group assignment, made the analysis of the behaviors.

At the end of each experiment, Evans blue dye (0.1%, 5 mg/Kg) was injected systemically (via penile vein) in order to confirm the TMJ injection site at post-mortem, as previously described [25] by the visual examination of formalin-induced plasma extravasation of Evans blue dye bond to plasma protein.

Drug treatments:

In order to evaluate the role of serotoninergic systems and endogenous opioids in nociceptive changes induced by stress, the serotonin-selective reuptake inhibitor (fluoxetine 10 mg/Kg) and the opioid agonist (morphine 1-5 mg/Kg) were administered before the nociception tests in some rats submitted to chronic stress as described above. Immediately after the last stress session (control group was handled as described above), the rats were injected i.p. with fluoxetine 10 mg/Kg [26], morphine 1.0 mg/Kg [27], 5.0 mg/Kg [28] or saline (n= 6/group) 30 min before the administration of formalin 1.5% into the TMJ. Morphine sulfate and fluoxetine hydrochloride (SIGMA) were dissolved in 0.9% saline and administered i.p. immediately after the last stress session.

Statistical analyses:

Statistical analyses of plasma corticosterone and ACTH data were made using the Mann-Whitney test (control vs. stressed) and Kruskal-Wallis (between stressed groups). The data were previously transformed to square-root or log, as indicated by PROCLAB-program

SAS (version 8.2 for windows). Since collected data about anxiety behavior didn't show normal distribution, the percentage of open-arm entries (100 X open/total) and the time spent in the open arms were calculated and analyzed by Mann-Whitney test. The sum of rubbing and flinching responses exhibited by each animal was computed. The comparison between two groups was made by Student's t-test. The comparison of more than two groups was made by two-way analysis of variance (ANOVA). The values for hormonal and nociceptive assays are given as mean +/- standard error of the mean (SEM). The data of anxiety behavior are expressed as median. A level of 5% was taken as evidence of statistical significance. Data were analyzed using SAS (version 8.2 for windows) by Institute Inc., Cary, NC, USA-licensed to Universidade Estadual de Campinas.

Results

Effects of stress procedures on plasma corticosterone and ACTH levels:

This experiment was carried out to define the efficacy of restraint in inducing stress-like hormonal modifications. There was a significant increase in plasma corticosterone levels after the various stress protocols used (Fig. 1; Mann-Whitney test, $p<0.05$). This increase was lower after sub-chronic and chronic stress than after acute stress for 30 min (Fig.1; Kruskal-Wallis, $p<0.05$).

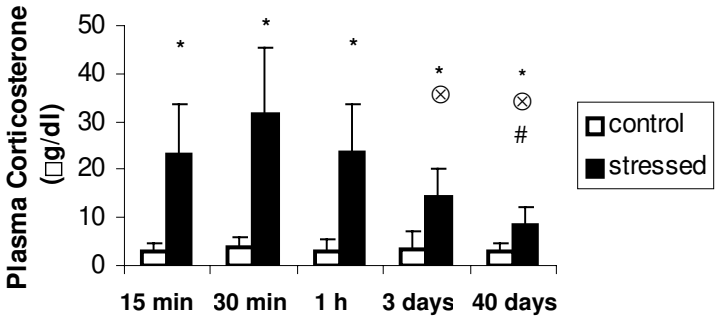

Fig 1. Plasma corticosterone level after the various stress procedures. Each data point represents mean ± SEM from 8 rats. The vertical bars indicate the standard error of the means. Data were analyzed using Mann-Whitney test and Kruskal-Wallis. (*) $p<0.05$ compared to respective control groups. (⊗) $p<0.05$ compared to acute stress 30 min. (#) $p<0.05$ compared to acute stress 15 min.

The increase in plasma ACTH levels was statistically significant for all acute groups tested (Fig. 2; Mann-Whitney test, $p<0.05$). There was no statistical difference between sub-chronic and chronic groups when compared with their respective control groups (Fig.2; Mann-Whitney test, $p<0.05$).

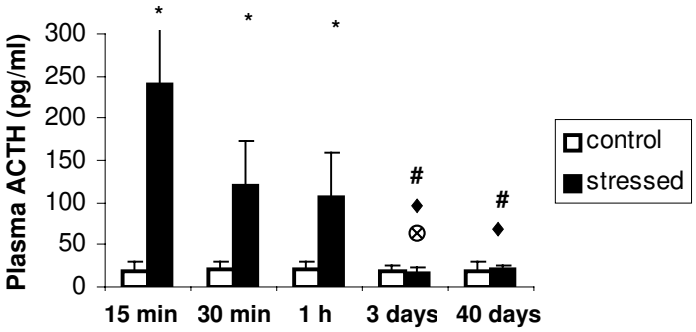

Fig 2. Plasma ACTH level after the various stress procedures. Each data point represents mean ± SEM from 8 rats. The vertical bars indicate the standard error of the means. Data were analyzed using Mann-Whitney test and Kruskal-Wallis. (*) $p<0.05$ compared to control groups. (#) $p<0.05$ compared to acute stress 15 min. (♦) $p<0.05$ compared to acute stress 30 min. (⊗) $p<0.05$ compared to acute stress 60 min.

Effects of stress procedures on the anxiety levels:

Figure 3 and Figure 4 show the effect of stress procedures on conventional anxiety indexes in the elevated plus-maze test. Stressed groups showed lower percentage of open arm entries and also of time spent in open arms when compared with their respective control groups (Fig 3 and Fig 4; Mann-Whitney test, $p<0.05$). No changes in the absolute number of entries in the closed arms were observed (data not shown).

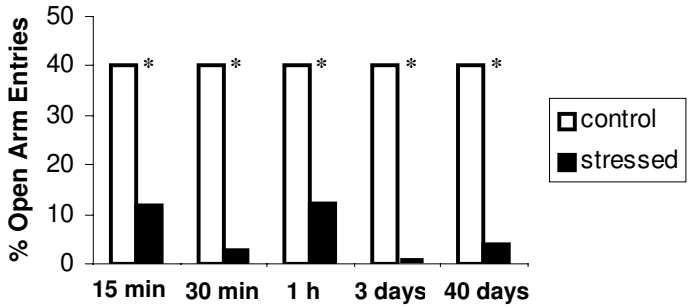

Fig 3. Effects of the various stress procedures on the percentage of entries in open arms. Bars represent the median. Number of subjects was set as N=10/group. (*) Indicates a significant difference from the respective control ($p<0.05$).

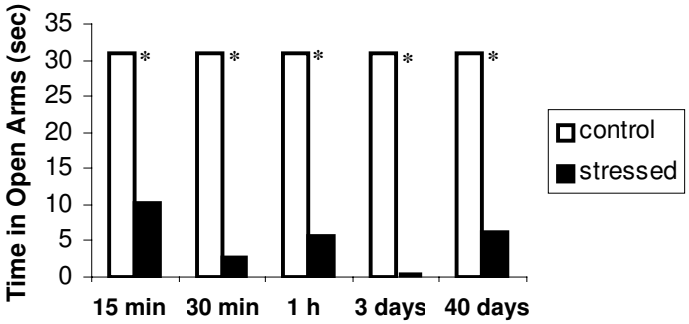

Fig 4. Effects of the various stress procedures on the time spent in open arms. Bars represent the median. Number of subjects was set as n=10/group. (*) Indicates a significant difference from the respective control (p<0.05).

Effects of stress procedures on the nociceptive responses induced by the TMJ formalin test: Results are shown in Figure 5. Immediately after the last restraint session (1 h /40 days), the chronically-stressed animals were hyperalgesic. The increase in the nociceptive behavioral responses was statistically significant (p<0.05, *t*-test) when the control group was compared with the stressed group. There was no statistical difference between the control groups (non-stressed) and the acute (15 min, 30 min or 60 min) and sub-chronic restraint groups (Fig 5).

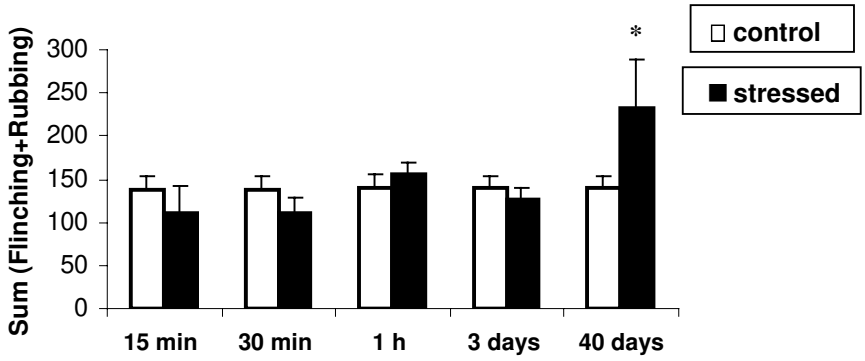

Fig 5. Sum of flinching and rubbing behaviors recorded in formalin-treated animals (50 µl, 1.5%) previously submitted to stress procedures (n=6/group) or left undisturbed in their home cage (n=6/group). Each column represents the mean. Error bars indicate the SEM. Data were analyzed using Student`s t-test. No significant differences were found in nociceptive responses for control vs. acute stressed groups (15 min, p=0.1571), (30 min, p=0.0754) and (1 h, p=0.1247). There was no statistical difference between sub-chronic and its respective control group (p=0.2149). (*) Indicates a significant between chronic and its respective control group (p<0.05).

Effect of fluoxetine on nociception in repeatedly-stressed and control rats:

Results are shown in Figure 6. ANOVA revealed difference between groups [F(1,20)=8.45; p=0.0087), drugs [F(1,20)=90.07; p<0.0001] and a significant interaction between group vs. Drug [F(1,20)=7.95; p=0.0106]. The administration of fluoxetine 10 mg/Kg 30 min prior to the TMJ formalin test produced a significant reduction in nociceptive behavioral responses both in control (p<0.001) and stressed rats (p<0.0001). The magnitude of the reduction in nociceptive responses was higher in stressed group (79,3%) than in control group (68%) (Fig 6).

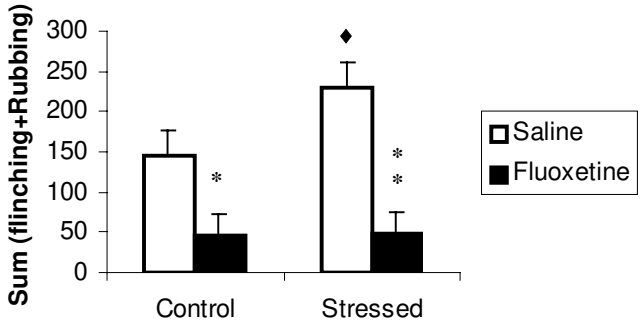

Fig 6. Effects of fluoxetine or saline on formalin-treated animals (50 µl, 1.5%) previously submitted to chronic restraint stress (*n*=6/group) or left undisturbed in their home cage (*n*=6/group). Each column represents the mean. Error bars indicate the SEM. (*) Indicates significant difference compared with the saline group (*p*=0.0001, t-test). (**) Indicates significant difference compared with the saline group (*p*<0.0001, t-test). (♦) Indicates significant difference between stressed and control rats (*p*=0.0006, t-test).

Effect of morphine on nociception in repeatedly-stressed and control rats:

Results referring to the analgesic effect of morphine are shown in Fig. 7. ANOVA revealed difference between groups [F(1,30)=53.54; p<0.0001), drugs [F(2,30)=35.94; p<0.0001] and a significant interaction between stress and morphine [F(2,30)=10.88; p=0.003]. Pos-hoc tests (Tukey) revealed that morphine administration produced a significant reduction of nociceptive behavioral responses in the control group (non-stressed). Morphine 1 mg/Kg reduced the nociceptive responses 30 min after the administration (*p*<0.05), and morphine 5

mg/Kg also had this effect (p<0.05). In the stressed group, morphine had an effect only at the dose of 5 mg/Kg (p<0.05) when compared to the saline group.

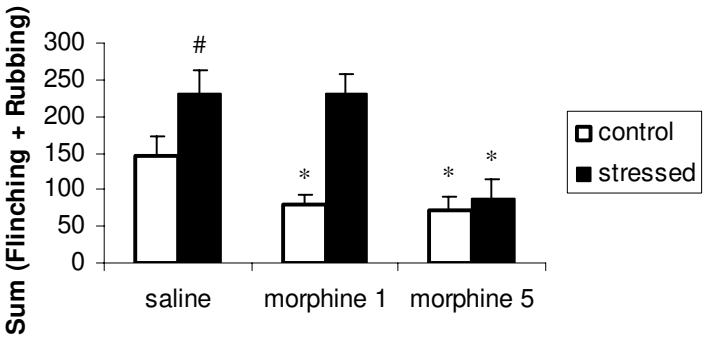

Fig 7. Sum of nociceptive responses to morphine (1 or 5 mg/Kg, i.p.) or saline after 40 days chronic restraint stress. White bars: control groups (n=6/group); Black bars: stressed groups (n=6/group). Each column represents the mean. Error bars indicate the SEM. (*) Significant difference between saline vs. morphine (p<0.05, ANOVA + Tukey). (#) Significant difference between control vs. stressed rats (p<0.05, ANOVA + Tukey).

Discussion

In both clinical and experimental settings, anxiety and the experience of pain are sometimes found to be positively related. It has been hypothesized that anxiety increases pain through the release of catecholamines, peripherally sensitizing or even stimulating nociceptors [29]. This idea is supported by research on sympathetically maintained pain (SMP), a chronic pain state that can be alleviated by sympathetic block or sympathectomy [30]. This is in contrast with Bolles and Fanselow (1980) [31], who postulated the view that anxiety inhibits pain through the release of endogenous opioids. Their view is also supported by some studies with humans [32, 33]. Clearly, research on the relationship between anxiety and pain so far has not led to unequivocal conclusions and underlying mechanisms are not fully understood. These discrepancies are due to the fact that experimental manipulations may radically alter the outcome of any behavioral model of nociception.

Thus, it has become important to assess the effects of different manipulations on the experimental animals during the stress procedures. In this context, we have measured the plasma corticosterone and ACTH levels as well as the anxiety level after different stress procedures. A significant increase in plasma corticosterone level was observed after acute (15 min, 30 min, 60 min), sub-chronic (3 days) and chronic (40 days) restraint stress sessions, although the level of ACTH was not statistically different between sub-chronic and chronic groups when compared with their respective control groups. Moreover, as expected, the increase in corticosterone levels was lower after chronic and sub-chronic stress when compared to acute protocols. Considering the corticosterone level as an indication of stress, all restraint procedures were able to induce stress. The various stress protocols were also able to induce significant anxiety levels, as observed in the responses to the elevated plus-maze test. Since the measurement of anxiety may be influenced by locomotor activity, we examined this factor by recording the absolute number of closed-arm entries, considered a clear index of general motor activity [22]. The lack of difference in the number of closed-arm entries between the stressed (all protocols) and control rats (data not shown) indicated that the locomotor activity was not influenced by stress procedure. These findings indicated that the anxiogenic effect after stress protocols was indeed related to anxiety and not to the locomotor activity of the rats.

Interestingly, the acute protocols (15 min, 30 min and 60 min) did not reduce the nociceptive behavioral responses evoked by nociceptive chemical stimulation (formalin 1.5%) of the rats´ TMJ. King et al., 2003 [7] showed that acute stress diminishes reflex responses to nociceptive input while enhancing operant responding to the same stimuli (nociceptive thermal stimuli), suggesting that stress induced hyporeflexia can coexist with stress induced hyperalgesia. According to these findings, we speculate that a single restraint session did not induce an analgesic effect on rats submitted to the TMJ formalin test, which evokes nociceptive responses that have an organization different from those related to innate reflexes, for example tail flick response that can be modulated directly at spinal levels [7]. Moreover, the absence of stress-induced analgesia in our model may be related to the different site of formalin injection. The discrepancy between nociception models in their susceptibility to modulation by stress is evident not only in the present results, but also

in the partial and transient analgesic effects found in other studies employing the formalin test [34, 35, 36]. We have already showed that restraint stress can release endogenous opioids [37]. In this case, it was expected that acute stress would reduce formalin-induced nociception, a finding not observed in our study. We believe that, in our model, the effects of endogenous opioids were counterbalanced by the enhance in pain perception evoked by stress-induced-anxiety. Studies have shown that hyperalgesia is elicited by some experimental conditions [38, 39, 40]. In the present work, we have demonstrated that a single exposure to restraint stress (15 min, 30 min and 60 min) increased the level of anxiety evaluated by the elevated-plus-maze test. This factor could also be determinant in the absence of stress-induced-analgesia.

Also, the sub-chronic stress model was not able to induce nociceptive changes in the TMJ formalin test. Quintero et al., 2000 [41] observed that rats showed an increased thermal and chemical nociception after sub-chronic swimming stress. Again, we believe that the different site of formalin injection and the stress procedure were responsible for these different results. Indeed, TMJ inflammation results in more robust changes in central nervous system when compared to perioral inflammation [42].

In contrast to acute and sub-chronic stress, we observed that the chronically stressed animals showed an increase in nociceptive behavioral responses when compared with the control group (non-stressed). In agreement with our results, previous studies have also found that chronic stress can elicit hyperalgesia rather than hypoalgesia [2, 41, 27, 43]. Although many studies indicate that corticosterone [44,45] and ACTH [46] can reduce nociceptive processing, we suggest that the stress-induced hyperalgesia on TMJ formalin test was not due to the low levels of corticosterone and ACTH observed in the chronically stressed rats. In light of our finding that rats submitted to sub-chronic stress also showed low levels of corticosterone and ACTH with no alterations in nociceptive responses, it appears that the hyperalgesia on chronic stress was the result of long-term effects evoked by persistent stress and anxiety.

Changes in the activity of central serotoninergic systems might explain, at least in part, the bidirectional changes in nociception (analgesia and hyperalgesia) seen after different stress conditions. For example, after acute exposure to different types of adverse psychological or

physical stimuli, there is an increase in the extracellular concentrations of serotonin in several brain regions, especially in the raphe magnus [47]. Conversely, prolonged stress diminishes the efflux of serotonin in some brain structures known to be activated by stress, such as the amygdala and the lateral septum [48]. We suggested that the anxiety and stress can cause a deficit in the central serotoninergic transmission which produces a sensitization of central pain relay pathways. In this study, we observed that chronically restraint rats exhibited a significant increase in anxiety levels. Fluoxetine administrated 30 min before formalin had an analgesic effect analog to that of morphine observed in one of our studies [49]. We suggest that this effect was due to the analgesic properties of fluoxetine [50, 51]. First, although the reduction in nociceptive responses was more significant in the stressed group, fluoxetine also reduced the nociceptive responses in the control group (non-stressed). Second, previous studies have shown that an acute dose of fluoxetine had an anxiogenic effect in the elevated plus-maze [52, 53]. These results indicate that the reduction in nociceptive behavior observed in our study was due to fluoxetine-induced antinociception, which involves both central opioid and the serotoninergic pathways [50]. Schreiber et al., 2001 [54] found that fluoxetine relieved low back pain with efficacy similar to that of amitriptyline, and they suggested that fluoxetine could be an alternative for patients unable to tolerate the tricyclic antidepressants' side effects.

We question the possibility to generalize experimental findings to clinical settings, that is to say, it is early to affirm that fluoxetine could be effective to treat TMD patients, even because some studies related that 5-HT re-uptake inhibitors have been associated with tooth-clenching or tooth-grinding [55]. Future studies should evaluate the possibility of dentist in using fluoxetine to treat TMD patients. We also tested control and repeatedly restrained rats injected with morphine (1 and 5 mg/Kg) in the TMJ formalin test. Our results demonstrate that repeatedly stressed rats display decreased morphine effects on nociception compared to non-stressed controls. The tolerance of response to morphine observed in our study agrees with the hypothesis suggested by previous studies that chronic stress could modify the activity of opioid systems [for review, see 56].

Overall, these observations support the concept that several mechanisms may simultaneously influence pain perception, some increasing and some inhibiting pain. The

development of experimental models such as the present one may provide further information about the mechanisms involved in painful conditions and may be used to test the efficacy of drugs. Stress induced hyperalgesia appears to result, at least in part, from changes in serotoninergic and opioid systems. Continued research concerning the mechanisms of stress-induced hyperalgesia may be relevant to the study of the etiology of chronic pain disorders, like the temporomandibular disorder.

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IV-CONCLUSÕES

De acordo com os resultados do presente trabalho, concluiu-se que:

- Apesar dos diversos protocolos de estresse utilizados em nosso estudo terem sido capazes de alterar significativamente os níveis hormonais, bem como o comportamento de ansiedade, apenas os animais cronicamente estressados apresentaram aumento nas respostas nociceptivas (hiperalgesia) quando submetidos ao teste da formalina na ATM.
- No grupo de estresse crônico, ocorreu redução do efeito analgésico da morfina, indicando disfunção do sistema opióide em animais cronicamente estressados.
- A fluoxetina teve efeito analgésico tanto no grupo estressado (hiperalgésico) quanto no grupo controle (não-estressado), porém seu efeito foi maior no grupo estressado, indicando o envolvimento dos sistemas serotoninérgicos na hiperalgesia induzida pelo estresse.
- A hiperalgesia induzida pelo estresse pode resultar de alterações nos sistemas opióides e serotoninérgicos, as quais representam uma importante comprovação para a relação existente entre estresse e dor orofacial.

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ANEXOS

Certificado do Comitê de ética

Universidade Estadual de Campinas
Instituto de Biologia

UNICAMP

CEEA-IB-UNICAMP

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **705-1**, sobre **"A INFLUÊNCIA DO ESTRESSE SOBRE A NOCICEPÇÃO INDUZIDA PELA INJEÇÃO DE FORMALINA NA ATM DE RATOS"** sob a responsabilidade de **Profa. Dra. Maria Cecília Ferraz de Arruda Veiga / Gustavo Hauber Gameiro** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 05 de Agosto de 2004.

CERTIFICATE

We certify that the protocol nº **705-1**, entitled **"THE INFLUENCE OF STRESS ON NOCICEPTION INDUCED BY FORMALIN INJECTED IN RAT'S TMJ"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on August 5, 2004.

Campinas, 05 e Agosto de 2004.

Profa. Dra. Liana Verinaud
Presidente

Fátima Alonso
Secretária

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Confirmação de Envio do Artigo para Publicação (Capítulo 1)

14-Feb-2006

Dear Dr. Gameiro:

Your manuscript entitled "How do stressful experiences contribute to the development of orofacial pain?" has been successfully submitted online and is presently being given full consideration for publication in the 'Clinical Oral Investigations'.

Your manuscript ID is COI-02-06-0026.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your street address or e-mail address, please log in to Manuscript Central at http://mc.manuscriptcentral.com/coi and edit your user information as appropriate.

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Thank you for submitting your manuscript to the 'Clinical Oral Investigations'.

Sincerely,

Gottfried Schmalz

Editor-in-Chief

Confirmação de Publicação do Artigo (Capítulo 2)

Pharmacol Biochem Behav. 2005 Oct;82(2):338-44. Epub 2005 Oct 6.

The effects of restraint stress on nociceptive responses induced by formalin injected in rat's TMJ.

Gameiro GH, **da Silva Andrade A**, **de Castro M**, **Pereira LF**, **Tambeli CH**, **Ferraz de Arruda Veiga MC**.

Laboratory of Orofacial Pain, Department of Physiology, Faculty of Dentistry of Piracicaba, University of Campinas-Unicamp, Av. Limeira 901 C.P. 52, CEP 13414-900, Piracicaba, Sao Paulo, Brazil.

It has been reported that stress can alter nociception from superficial tissues, such as skin and subcutaneous region. However, the influence of stress on an experimental deep nociception model is not understood. In this study, the temporomandibular joint (TMJ) formalin test was used to evaluate the effects of acute and chronic restraint stress on nociceptive responses in rats. Animals were initially submitted to one session of acute restraint stress (1 h) or exposed to chronic stress (40 days-1 h/day). Then, animals were killed immediately to collect blood for hormonal determinations by radioimmunoassay, or submitted to the TMJ formalin test to evaluate nociception. Rats submitted to acute restraint presented a performance similar to unstressed controls in the TMJ formalin test, whereas chronically stressed rats showed an increase in nociceptive responses. After 40 days of restraint, morphine was injected i.p. (1, 5 mg/kg or saline). The stressed rats displayed decreased morphine effects on nociception compared to unstressed controls. These findings suggest that repeated stress can produce hyperalgesia, which is, at least in part, due to alterations in the activity of opioid systems. This model may help elucidate the underlying neural mechanisms that mediate the effects of repeated stress on orofacial pain.

PMID: 16213578 [PubMed - in process]

Confirmação de Aceite do Artigo (Capítulo 3)

Data: Fri, 02 Dec 2005 16:37:41 -0000
De: physiolbehav@psychiatry.uc.edu
Para: ggameiro@fop.unicamp.br
Assunto: Your Submission
Ms. Ref. No.: PHB-D-05-00114R1
Title: Nociception- and anxiety-like behavior in rats submitted to different periods of restraint stress from, Gustavo Hauber Gameiro, Paula Hauber Gameiro, Annicele da Silva Andrade, Lígia Ferrinho Pereira, Mariana Trevisani Arthuri, Fernanda Klein Marcondes and Maria Cecília Ferraz de Arruda Veiga
Physiology & Behavior

Dear Gameiro,

I am pleased to inform you that your manuscript referenced above has been accepted for publication in Physiology & Behavior.

Many thanks for submitting your fine paper to Physiology & Behavior. I look forward to receiving additional papers from you in the future.

With kind regards,

Stephen C. Woods
Editor-in-Chief
Physiology & Behavior

APÊNDICE

FIGURAS

Figura 1: Tubo plástico utilizado para realização da contenção

Figura 2: Local da punção para injeção de formalina na ATM

Figura 3: Câmara de observação utilizada para registro das respostas nociceptivas

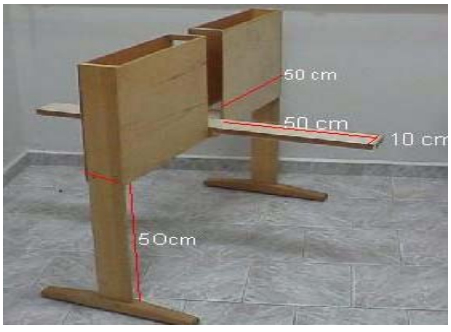

Figura 4: Labirinto utilizado para avaliação da ansiedade (teste do labirinto em cruz elevado)

TABELAS REFERENTES AOS VALORES INDIVIDUAIS DA AMOSTRA

Tabela 1 – Valores individuais do nível de corticosterona plasmática em animais submetidos a uma sessão de estresse agudo por 15 minutos.

Corticosterona Plasmática (μg/dl)		
Animal	**Grupo Controle (não-estressado)**	**Grupo Estressado**
1	0,7	18,9
2	5,5	20,8
3	1,0	15,6
4	3,2	22,8
5	2,3	25,9
6	4,8	34,6
7	4,3	26,6
8	1,3	21,3
Média ± EPM	2,89 ± 1,56	23,31± 10,15

Tabela 2– Valores individuais do nível de corticosterona plasmática em animais submetidos a uma sessão de estresse agudo por 30 minutos.

Corticosterona Plasmática (μg/dl)		
Animal	**Grupo Controle (não-estressado)**	**Grupo Estressado**
1	0,7	31,6
2	5,5	35,0
3	1,0	14,8
4	3,2	40,0
5	2,3	24,3
6	4,8	37,8
7	4,3	27,7
8	8,6	40,0
Média ± EPM	3,80 ± 2,00	31,40 ± 13,85

Tabela 3– Valores individuais do nível de corticosterona plasmática em animais submetidos a uma sessão de estresse agudo por 60 minutos.

Corticosterona Plasmática (μg/dl)		
Animal	**Grupo Controle (não-estressado)**	**Grupo Estressado**
1	0,7	18,5
2	4,8	34,8
3	0,7	22,6
4	2,3	14,9
5	0,7	14,1
6	9,4	27,6
7	0,8	21,5
8	4,3	33,9
Média ± EPM	2,96 ± 2,40	23,49 ± 10,16

Tabela 4– Valores individuais do nível de corticosterona plasmática em animais submetidos ao protocolo de estresse sub-crônico (1 h /3 dias).

Corticosterona Plasmática (μg/dl)		
Animal	**Grupo Controle (não-estressado)**	**Grupo Estressado**
1	0,8	18,4
2	0,3	15,1
3	9,2	3,3
4	8,6	15,1
5	7,2	11,1
6	0,7	10,8
7	0,7	9,7
8	0,9	31,1
Média ± EPM	3,55 ± 3,59	14,33 ± 6,00

Tabela 5– Valores individuais do nível de corticosterona plasmática em animais submetidos ao protocolo de estresse crônico (1 h /40 dias).

Corticosterona Plasmática (μg/dl)		
Animal	**Grupo Controle (não-estressado)**	**Grupo Estressado**
1	0,7	2,5
2	5,5	2,7
3	1,0	9,3
4	3,2	4,8
5	2,3	2,4
6	4,8	15,8
7	0,9	14,1
8	1,3	15,5
Média ± EPM	2,46 ± 1,53	8,39 ± 3,89

Tabela 6– Valores individuais do nível de ACTH plasmática em animais submetidos a uma sessão de estresse agudo por 15 minutos.

ACTH Plasmática (pg/ml)		
Animal	**Grupo Controle (não-estressado)**	**Grupo Estressado**
1	10,0	77,6
2	27,7	132
3	10,0	83,6
4	29,7	261
5	10,0	706
6	41,4	410
7	14,0	40,6
8	10,0	200
Média ± EPM	19,10 ± 10,38	238,85 ± 125,29

Tabela 7– Valores individuais do nível de ACTH plasmática em animais submetidos a uma sessão de estresse agudo por 30 minutos.

ACTH Plasmática (pg/ml)		
Animal	**Grupo Controle (não-estressado)**	**Grupo Estressado**
1	10,0	246
2	27,7	185
3	10,0	42
4	29,7	82,8
5	10,0	55,5
6	41,4	172
7	14,0	66,6
8	15,5	104
Média ± EPM	19,79 ± 9,86	119,24 ± 52,86

Tabela 8– Valores individuais do nível de ACTH plasmática em animais submetidos a uma sessão de estresse agudo por 60 minutos.

ACTH Plasmática (pg/ml)		
Animal	**Grupo Controle (não-estressado)**	**Grupo Estressado**
1	38,6	138
2	41,4	90,8
3	10,0	33,0
4	10,0	35,8
5	10,0	38,0
6	19,4	172
7	17,7	41,7
8	14,0	309
Média ± EPM	20,14 ± 9,93	107,29 ± 52,95

Tabela 9– Valores individuais do nível de ACTH plasmática em animais submetidos ao protocolo de estresse sub-crônico (1 h/3 dias).

ACTH Plasmática (pg/ml)		
Animal	**Grupo Controle (não-estressado)**	**Grupo Estressado**
1	17,7	15,0
2	27,0	29,4
3	19,4	10,0
4	15,5	13,7
5	17,6	10,2
6	38,6	10,0
7	10,0	14,2
8	10,6	25,0
Média ± EPM	19,55 ± 6,63	15,94 ± 6,27

Tabela 10– Valores individuais do nível de ACTH plasmática em animais submetidos ao protocolo de estresse crônico (1 h/40 dias).

ACTH Plasmática (pg/ml)		
Animal	**Grupo Controle (não-estressado)**	**Grupo Estressado**
1	10,0	21,6
2	27,7	16,8
3	10,0	22,2
4	29,7	14,7
5	10,0	20,0
6	41,4	18,4
7	10,6	18,9
8	10,0	25,4
Média ± EPM	18,68 ± 10,69	19,75 ± 6,36

Tabela 11 – Valores individuais do efeito desencadeado pelos diversos protocolos de estresse sobre a porcentagem de entrada nos braços abertos durante o teste do labirinto em cruz elevado.

Avaliação da ansiedade: % entrada nos braços abertos						
Animais (N=10/grupo)	**Grupo controle**	**Estresse 15 min.**	**Estresse 30 min.**	**Estresse 60 min.**	**Estresse 3 dias (1h/dia)**	**Estresse 40 dias (1h/dia)**
1	20	16,7	0,00	10,0	0,00	0,00
2	33,3	0,00	33,3	50,0	0,00	0,00
3	44,4	22,2	0,00	50,0	0,00	0,00
4	30,0	12,5	5,90	0,00	11,1	13,3
5	44,4	11,1	0,00	20	42,9	26,7
6	42,9	42,9	20,0	14,3	12,5	40,0
7	45,5	0,00	0,00	16,7	0,00	0,00
8	33,3	11,1	0,00	0,00	0,00	33,3
9	50,0	0,00	25,0	0,00	0,00	7,70
10	37,5	21,4	33,3	0,00	8,30	0,00
Mediana	40,20	11,8	2,95	12,15	0,00	3,85

Tabela 12 – Valores individuais do efeito desencadeado pelos diversos protocolos de estresse sobre o tempo de permanência nos braços abertos durante o teste do labirinto em cruz elevado.

Avaliação da ansiedade: tempo permanência nos braços abertos (segundos)						
Animais (N=10/grupo)	**Grupo controle**	**Estresse 15 min.**	**Estresse 30 min.**	**Estresse 60 min.**	**Estresse 3 dias (1h/dia)**	**Estresse 40 dias (1h/dia)**
1	10,0	7,40	0,00	9,53	0,00	0,00
2	10,03	0,00	22,74	19,78	0,00	0,00
3	30,0	25,0	0,00	29,84	0,00	0,00
4	19,72	13,05	10,22	0,00	26,23	33,29
5	88,2	18,72	0,00	15,16	55,08	40,54
6	31,57	29,7	9,94	1,84	23,40	12,41
7	71,84	0,00	0,00	32,13	0,00	0,00
8	59,75	2,06	0,00	0,00	0,00	51,97
9	82,34	0,00	5,12	0,00	0,00	14,09
10	30,19	33,5	27,31	0,00	11,78	0,00
Mediana	30,88	10,23	2,56	5,69	0,00	6,21

Tabela 13 – Valores individuais da soma dos comportamentos nociceptivos [coçar (CO)+levantar rapidamente a cabeça (LC)] desencadeados pela injeção de formalina na ATM após estresse por contenção durante 15 minutos.

Soma dos comportamentos (CO + LC)		
Animal	**Grupo Controle**	**Grupo Estressado**
1	148,45	124,07
2	128,42	49,34
3	171,71	138,54
4	134,36	129,55
5	116,74	144
6	123,16	73
Média ± EPM	137,14 ± 15,29	109,75 ± 32,39

Tabela 14 – Valores individuais da soma dos comportamentos nociceptivos [coçar (CO)+levantar rapidamente a cabeça (LC)] desencadeados pela injeção de formalina na ATM após estresse por contenção durante 30 minutos.

Soma dos comportamentos (CO + LC)		
Animal	**Grupo Controle**	**Grupo Estressado**
1	148,45	124
2	128,42	130,67
3	171,71	120,32
4	134,36	112
5	116,74	117,14
6	123,16	59,05
Média ± EPM	137,14 + 15,29	110,53 + 17,16

Tabela 15 – Valores individuais da soma dos comportamentos nociceptivos [coçar (CO)+levantar rapidamente a cabeça (LC)] desencadeados pela injeção de formalina na ATM após estresse por contenção durante 60 minutos.

Soma dos comportamentos (CO + LC)		
Animal	**Grupo Controle**	**Grupo Estressado**
1	128,42	116,74
2	134,36	124,69
3	116,74	148,79
4	150,22	108
5	148,45	122
6	158,23	144,05
Média ± EPM	139,40 ± 12,90	127,38 ± 12,69

Tabela 16 – Valores individuais da soma dos comportamentos nociceptivos [coçar (CO)+levantar rapidamente a cabeça (LC)] desencadeados pela injeção de formalina na ATM após estresse por contenção durante 3 dias (1h/dia).

Soma dos comportamentos (CO + LC)		
Animal	**Grupo Controle**	**Grupo Estressado**
1	158,23	137
2	128,42	149,64
3	171,71	148,18
4	134,36	177,62
5	116,74	158,63
6	123,16	168,92
Média ± EPM	138,77 ± 17,47	156,67 ± 11,73

Tabela 17 – Valores individuais da soma dos comportamentos nociceptivos [coçar (CO)+levantar rapidamente a cabeça (LC)] desencadeados pela injeção de formalina na ATM após estresse por contenção durante 40 dias (1h/dia).

Soma dos comportamentos (CO + LC)		
Animal	**Grupo Controle**	**Grupo Estressado**
1	128,42	241,58
2	134,36	331,2
3	116,74	186,63
4	150,22	291,03
5	148,45	150,71
6	158,23	189
Média ± EPM	139,40 ± 12,90	231,69 ± 56,25

Tabela 18 – Valores individuais da soma dos comportamentos nociceptivos [coçar (CO)+levantar rapidamente a cabeça (LC)] desencadeados pela injeção de formalina na ATM após estresse por contenção durante 60 minutos e administração de salina ou naloxona (10 mg/Kg).

Soma dos comportamentos (CO + LC)		
Animal	**Salina**	**Naloxona**
1	112,26	209,17
2	119,87	120,7
3	137	268,1
4	87,72	200,69
5	124	114
6	158	171,5
Média ± EPM	123,14 ± 16,53	180,69 ± 45,29

Tabela 19 – Valores individuais da soma dos comportamentos nociceptivos [coçar (CO)+levantar rapidamente a cabeça (LC)] desencadeados pela injeção de formalina na ATM após administração de salina ou morfina (1 e 5 mg/Kg).

Soma dos comportamentos (CO + LC)			
Animal (Controle)	**Salina**	**Morfina 1 mg/Kg**	**Morfina 5 mg/Kg**
1	145,41	83	72
2	131,90	79	106,63
3	208,80	92,06	55,68
4	188,09	105	53,88
5	132,85	47,6	89,31
6	219,40	76,2	48,53
Média ± EPM	171,07 ± 39,24	80,48 ± 12,88	71,01 ± 18,31

Tabela 20 – Valores individuais da soma dos comportamentos nociceptivos [coçar (CO)+levantar rapidamente a cabeça (LC)] desencadeados pela injeção de formalina na ATM após estresse por contenção durante 40 dias (1h/dia) e administração de salina ou morfina (1 e 5 mg/Kg).

Soma dos comportamentos (CO + LC)			
Animal (Estressado)	**Salina**	**Morfina 1 mg/Kg**	**Morfina 5 mg/Kg**
1	232,2	208,5	44
2	246,6	221,23	91,71
3	200,57	232,86	144,11
4	173,42	296,31	77,74
5	225,87	240	69,31
6	306	187,2	103,2
Média ± EPM	230,78 ± 30,82	231,02 ± 25,37	88,35 ± 24,66

Tabela 21 – Valores individuais da soma dos comportamentos nociceptivos [coçar (CO)+levantar rapidamente a cabeça (LC)] desencadeados pela injeção de formalina na ATM após estresse por contenção durante 40 dias (1h/dia) e administração de salina ou fluoxetina (10 mg/Kg).

Soma dos comportamentos (CO + LC)				
Animal	**Grupo Controle (não estressado)**		**Grupo Estressado**	
	salina	Fluoxetina	Salina	fluoxetina
1	171,15	93	232,2	88,1
2	165,14	30,4	246,6	52
3	158,07	52	200,57	3
4	123,81	75	173,42	46
5	165,16	10	225,87	82
6	90,34	18	306	15
Média ± EPM	145,61 ± 25,69	46,40 ± 26,93	230,78 ± 30,82	47,68 ± 26,35

Tabela 22 – Valores individuais do efeito do estresse por contenção durante 40 dias (1h/dia) sobre o comportamento de coçar a região orofacial desencadeado pela injeção de salina na ATM.

Comportamento de coças a região orofacial (segundos)		
Animal	**Grupo Controle**	**Grupo Estressado**
1	23,71	43,05
2	49,4	0
3	28,28	11,02
4	35,19	71,02
5	50,25	18,9
6	20,46	52
Média ± EPM	34,55 ± 10,40	32,67 ± 22,69